FIA-automated system used to electrochemically measure nitrite and its interfering chemicals through a 1-2 DAB / Au electrode: gain of sensitivity at upper potentials

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Abstract. The measurement of nitrite and its interfering-chemicals (paracetamol, ascorbic acid and uric acid) was performed employing a Flow-injection Analysis (FIA) system, which was automated using solenoid valves and air-pump. It is very important to quantify nitrite from river water, food and biologic fluids due to its antibacterial capacity in moderated concentrations, or its toxicity for human health even at low concentrations (> 20 µmol L⁻¹ in blood fluids). Electrodes of the electrochemical planar sensor were defined by silk-screen technology. The measuring electrode was made from gold paste covered with 1-2 cis Diaminobenzene (DAB), which allowed good selectivity, linearity, repeatability, stability and optimized gain of sensitivity at 0.5 V_{Ag/AgCl Nafion®117} (6.93 µA mol⁻¹ L mm⁻²) compared to 0.3 V_{Ag/AgCl Nafion®117}. The reference electrode was obtained from silver/palladium paste modified with chloride and covered with Nafion® 117. The auxiliary electrode was made from platinum paste. It was noteworthy that nitrite response adds to the response of the studied interfering-chemicals and it is predominant for concentrations lower than 175 µmol L⁻¹.

Keywords: nitrite measurement, 1-2 DAB, amperometric sensor and electrochemical potential

1. Introduction

From the 70’s and 80’s until now, the micro-fabrication, silkscreen and microelectronic technologies have been employed to fabricate nitrite biosensor and electrochemical sensors [1-3]. In recent years, the electrochemical techniques have been largely employed in sensors due to the easy setup, versatility in modification of the surface electrodes, and, especially, because they allow direct amperometric measurements with lower detection limits [2,4].

The need for quantitative measurement of nitrite (NO₂⁻) has increased due to its toxicity potential for human health (if > 21.7 µmol l⁻¹ in potable water) [5] and environmental security (< 21.7 µmol l⁻¹ in river water) [6]. Nitrites can react with amines in stomach forming nitrosamines, which are known to be carcinogenic [4,7,8].

Nitrite concentrations in blood are in the range from undetectable to 20 µmol l⁻¹ [9]. If nitrite is administrated at low levels (< 1 µmol l⁻¹), it can significantly reduce cardiac infarct size by approximately 50-67% [10]. On the other hand, if nitrite is present at high levels, it can react with hemoglobin, forming methemoglobin (reaction 1), which has no oxygen carrying ability [11,12].

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Nitrite, as key species in the global nitrogen cycle [13,14], is present in soils, drinking and river waters, foods and physiological systems. Also, it is employed as food preservative against poisoning by microorganisms such as *clostridium botulinum* and it appears in water during chlorination.

Nitrite’s anion is of interest as one Nitric Oxide (NO) precursor in biological systems, and its indirect detection has recently been investigated since it is an important element associated to some physiological events, like vasodilatation, neurotransmission [15], inflammation and cell proliferation. Reaction 2, as follows, illustrates the formation of NO, which may occur in the human body [4].

\[
4{\text{NO}}_{2}^{-} + 4HbO_2 \rightarrow 4{\text{NO}}_{3}^{-} + 2Hb_2O + O_2
\]  

The 1-2 cis Diaminobenzene (1-2 DAB) film was chosen as suggested by previous studies [2,16,17] that had shown an increase of selectivity to the nitrite and a decrease of the interfering-chemicals signals for uric acid (UA), ascorbic acid (AA) and paracetamol (PA) at maximum concentrations used as therapeutic doses in blood of a patient [17] 0.52 mmol l⁻¹, 0.11 mmol l⁻¹ and 0.15 mmol l⁻¹, respectively.

In addition, we have studied the sensor integrated to an Automated Flow-injection Analysis (FIA-automated) System in order to improve repeatability and stability [18]. In Intensive Therapy Unities, it is important to control the nitrite concentration to avoid patient intoxication due to diet, accelerated metabolism or medicaments.

2. Procedures and Methods

2.1. Fabrication of the Sensor

The proposed sensor, composed by three electrodes, was silk-screened onto an alumina substrate of (17 x 43) mm. The measuring electrode was made with DuPont® 5142 gold paste, the reference electrode, with Pd/Ag DuPont® 6146 paste modified for the chlorination and covered with a Nafion® 117 membrane and the auxiliary electrode, with ESL 5545-LS platinum paste. The geometric areas were 4.5 ± 0.2 mm², 4.2 ± 0.7 mm² and 59.8 ± 0.7 mm²; for measuring, reference and auxiliary electrodes, respectively. These can be observed in figure 1. In addition, Au paste and DuPont® 9615 were utilized to define connection lines / contacts and electric isolation, respectively.

![Figure 1. Schematic of the planar sensor with the respective electrodes.](image-url)
2.2. Cleaning of the Electrodes

After the fabrication, it was necessary to activate the electrodes surface to guarantee the increase of sensitivity. In other words, a standard cleaning procedure (physic, chemical and electrochemical) was performed as reported in [17] to clean the gold surface. At first, the surface was polished with 0.05 µm gel alumina (thirty 8-shape movements), then, it was rinsed in acetone and alcohol, and, finally, Cyclic Voltammetry (CV) was performed by applying 90 voltage cycles from -0.9 to +0.9 V$_{Ag/AgCl}$ 3 M KCl (scanning rate of 0.3 V s$^{-1}$) in a 0.1 mol l$^{-1}$ H$_2$SO$_4$ solution. The results of cleaning allowed maximum repeatability and reproducibility of the measuring electrode.

It was noteworthy that black spots on the electrode surface were observed under optical microscopy before been cleaned and that they were removed after cleaning, as shown in figure 2.

![Figure 2. Optical microscopy images of the gold electrode surface, a) before cleaning procedures and b) after chemical cleaning procedures.](image)

Normally, during the fabrication process of the electrochemical sensors or biosensors, it is convenient to deposit a conductor film above the working electrode surface to decrease the influence of the interfering elements and to increase the measured signal. In our work, 1-2 cis Diaminobenzene (DAB) was chosen because it fulfils this requirement and due to its previous use [16,18-20] with selectivity for nitrite. In addition, we have studied this membrane with Automated Flow-Injection Analysis (FIA) Technique in order to improve repeatability, reproducitively, stability and linearity [18].

2.3. Characterization

CV characterization of the electrodes allows us to establish functionality based on the current measurement. The CV technique using a PalmSens voltammetric instrument was employed to obtain the effective area in solution for the bare-gold measuring electrode, and to evaluate its stability.

![Figure 3. Cyclic voltammograms to extract peak current before and after cleaning, and, so, to calculate the effective area of the measuring electrodes.](image)

![Figure 4. Reversibility curve of the Ag/AgCl Nafion® 117 film as the reference electrode.](image)
A solution of 0.1 mol l⁻¹ potassium ferricyanide in 0.5 mol l⁻¹ KCl was used with the following parameters: 6 cycles of a voltage ramp varying from +0.8 to -0.2 V <sub>Ag/AgCl 3M KCl</sub> with scanning rate of 0.1 V s⁻¹ (figure 3).

The effective area of 4.33 mm² for the measuring electrode was obtained from Randles-Sevcik’s equation and with the aid of the CV in figure 3 [18,21]. One observes a decrease of 3.7 % in relation geometric area due to electric isolation layer around gold that reduces area.

To obtain a AgCl film on the reference electrode, a chlorination reaction was performed in a 0.1 mol l⁻¹ HCl solution at a fixed potential of 0.86 V <sub>Ag/AgCl 3M KCl</sub> (0.5 mA cm⁻²) during ~34 min (integrated charge of 950 mC for 5 µm of AgCl) followed by a confinement in serum physiologic (NaCl 0.9% and pH = 7.3) during three days.

Following, a 3 mol l⁻¹ NaCl solution was employed to perform the tests of reversibility of the reference electrode. First, the Ag/AgCl junction was stabilized by using a voltage ramp from -0.3 to +0.3 V <sub>Ag/AgCl 3M KCl</sub> (50 cycles with scanning rate of 0.3 V s⁻¹); then, the reversibility test was performed for a voltage ramp from -0.5 to +0.5 V <sub>Ag/AgCl 3M KCl</sub> (6 cycles with scanning rate of 0.1 V s⁻¹), as shown in figure 4.

![Figure 5. Potential evolution test as a function of the time of Nafion® 117.](image)

![Figure 6. Electrodeposition of the 1-2 DAB film by CV (50 cycles from 0 to +0.65 V <sub>Ag/AgCl 3M KCl</sub> with scanning rate of 0.1 V s⁻¹).](image)

Figure 5 shows another test. In this case, the voltage behavior of the Ag/AgCl Nafion® 117 film. The resulting average electrical potential was 98 ± 3 mV <sub>Ag/AgCl 3M KCl</sub> with a degradation of 29.6 µV h⁻¹.

2.4. Surface Modification of the gold electrode

5 mmol l⁻¹ 1-2 DAB was added to 0.05 mol l⁻¹ a phosphate buffer (pH = 7.2), which was purged during 30 min in nitrogen gas. Following, the 1-2 DAB film was electrodeposited over the gold surface using CV in PalmSens (50 cycles from 0 to +0.65V <sub>Ag/AgCl 3M KCl</sub> with scanning rate of 0.1 V s⁻¹) as shown in figure 6.

1-2 DAB film electro-activated onto gold surface contributes to decreasing the reactivity and increasing the stability and repeatability of the measured FIA signals.

2.5. FIA-automated System

The FIA system is composed of solenoid valves, discard, analysis-cell, air-pump, electrical and fluidic connections, PalmSens potentiostat and analytes. The experimental set-up is shown in figures 7 and 8. It is noteworthy the analysis-cell (at the lower right corner) and control computer (at the upper left corner) in figure 8.
The physiologic serum (NaCl 0.9 % and pH = 7.3) was employed as solvent or as cleaning and carrier solution during the measurements. The concentrations performed for nitrite were 50, 100 and 250 µmol l⁻¹, and the interfering-chemicals were UA (0.55 mmol l⁻¹ diluted in 0.1 mol l⁻¹ NaOH), AA (0.11 mmol l⁻¹) and PA (0.17 mmol l⁻¹) with concentration chosen at the maximum therapeutic doses in blood of a patient [17].

Figure 7 shows the operational scheme of the FIA-automated System, developed for measurement of nitrite and their interfering-chemicals.

![Figure 7](image)

**Figure 7.** FIA-automated composed by the solenoid valves (V₁, V₂, V₃ e V₅) and flow control valves (V₆, V₇, V₈ e V₉) for injection of analysis solutions (S₁, S₂ e S₃), cleaning solution (S₆), carrier solution (S₅), sensor, discard (D) and FIA signal.

It is identified in the scheme of the FIA-automated System:

- a, b, c, d: dots of air confluence.
- SC: Carrier Solution.
- SL: Cleaning Solution.
- S₁: Analyte Solution 1.
- S₂: Analyte Solution 2.
- S₃: Analyte Solution 3.
- Vₛ₁, Vₛ₂, Vₛ₃ e Vₛ₅ off, it flows S₅.
- Vₛ₁: Solenoid Valve 1 on, it injects S₁.
- Vₛ₂: Solenoid Valve 2 on, it injects S₂.
- Vₛ₃: Solenoid Valve 3 on, it injects S₃.
- V₅: Solenoid Valve of cleaning on, it flows S₆ by action of gravitational force.
- V₆: Valve to set flow S₅.
- V₇: Valve to set flow S₆.
- V₈: Valve to set flow S₇.
- V₉: Valve to set air flow, it allows to drain air excess.
- CA: air compressor employed for pumping by pressure, carrying of solutions.
Figure 8 is a picture of the FIA-automated System indicating the solenoids and flow valves to control the nitrite, interfering-chemicals solutions, the cleaning and the carrier solutions during the FIA process.

![Figure 8](image)

**Figure 8.** Automated FIA system to measure nitrite and interfering-chemicals.

The picture of the experimental setup in figure 8 also shows analysis cell, air pump, analytes, analysis-cell, fluidic and electrical connections and discard. This equipment was designed to simulate blood flow utilizing physiologic serum (NaCl 0.9 % and pH = 7.3) as carrier and cleaning solution in the FIA-automated system.

2.6. Determination of the electrochemical potentials for FIA

Differential Pulse Voltammetry (DPV) technique [22] was employed to obtain the electrochemical potentials to be used in FIA in order to obtain the concentrations of nitrite, AA, UA and PA at constant potentials. Figure 9 illustrates the start and the peak potential for nitrite reduction.

![Figure 9](image)

**Figure 9.** Standard DPV measurement showing the start and the peak current and potential for nitride reduction.

Table 1 shows the peak current, the start and the peak potentials of nitrite, AA, UA and PA for concentrations of 2.00 mmol l$^{-1}$, 0.44 mmol l$^{-1}$, 2.00 mmol l$^{-1}$ and 0.68 mmol l$^{-1}$, respectively. The concentrations of AA, UA and PA were chosen as the maximum therapeutic doses in blood of a
patient, here defined as the worst condition to be achieved in order to allow the measure of the nitrite concentration without the interference of AA, UA and PA.

Based on the peak currents presented in Table 1 and aiming to perform FIA process at constant potentials, 0.3 and 0.5 V$_{\text{Ag/AgCl Nafion}^\circ 117}$ were arbitrarily chosen to allow neglecting the measured current for AA, UA and PA compared to the corresponding for the nitrite concentration.

| Metabolites         | Concentration Mmol L$^{-1}$ | Peak Potential V$_{\text{Ag/AgCl Nafion}^\circ 117}$ | Start Potential of V$_{\text{Ag/AgCl Nafion}^\circ 117}$ |
|---------------------|-----------------------------|---------------------------------|---------------------------------|
| Nitrite             | 2.00                        | 0.880                           | 0.500                           |
| Ascorbic Acid (AA)  | 0.44                        | 0.292                           | 0.172                           |
| Uric Acid (UA)      | 2.00                        | 0.472                           | 0.352                           |
| Paracetamol (PA)    | 0.68                        | 0.602                           | 0.372                           |

3. Results and Discussion

Using the procedure as described in the experiment, FIA measurements were performed. Graphs “a” and “b” in figure 10 correspond to the FIA response of the interfering-chemicals (AA, PA and UA). It was noteworthy that AA and paracetamol presented similar pulsed-current signals at 0.3 V$_{\text{Ag/AgCl Nafion}^\circ 117}$ above the nitrite signal in figure 10(c). On the other hand, for 0.5 V$_{\text{Ag/AgCl Nafion}^\circ 117}$, AA, PA and UA signals were lower than the nitrite signal compare figure 10(b) with figure 10(d)). It means that the interfering-chemicals can be neglected compared to nitrite.

From FIA, the curves of sensitivity shown in figure 11 could be plotted and the slope could be extracted and normalized concerning to the effective area of 4.33 mm$^2$. In addition, current signals of the interfering-chemicals compared to the nitrite were also evaluated from FIA (figure 12).
The result above indicates that the nitrite signal is higher than the interfering-chemicals for 0.5 V\textsubscript{Ag/AgCl} Nafion\textsuperscript{117}. On the other hand, for 0.3 V\textsubscript{Ag/AgCl} Nafion\textsuperscript{117} the nitrite signal is lower than the signals of the interfering-chemicals (AA, UA and PA). As a result, it is concluded that it is feasible to obtain the concentration or nitrite by FIA at 0.5 V\textsubscript{Ag/AgCl} Nafion\textsuperscript{117} in a selective way without substantial influence of UA, PA and AA.

4. Conclusions
The presented results indicated linearity, repeatability, stability and selectivity for nitrite measurement. It was demonstrated the feasibility of obtaining the concentration of nitrite by FIA at 0.5 V\textsubscript{Ag/AgCl} Nafion\textsuperscript{117}, in a selective way without substantial influence of UA, PA and AA. The obtained sensitivity for nitrite was on order of 6.93 µA mol\textsuperscript{-1} L mm\textsuperscript{-2} and 7.28 nA mol\textsuperscript{-1} L mm\textsuperscript{-2} for FIA performed at 0.5 V\textsubscript{Ag/AgCl} Nafion\textsuperscript{117}, and 0.3 V\textsubscript{Ag/AgCl} Nafion\textsuperscript{117}, respectively.

5. References
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