Sensing and Bio-Sensing Research 49 (2025) 100860

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

Point-of-care testing of nitrite in oral medicine - Application and validation of an enzymatic biosensor in human saliva

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ARTICLE INFO

Keywords:
Periodontal disease
Saliva
Nitrite
Biosensors
Point-of-care test

ABSTRACT

Periodontal disease (PD) is a chronic inflammatory condition triggered by bacterial biofilms and progresses through two main stages: gingivitis and periodontitis. The existing diagnostic methods remain time-consuming and require clinical expertise. Salivary nitrite, a stable end-product of nitric oxide produced during inflammation, has emerged as a promising biomarker for PD. However, its reliable quantification in saliva typically relies on laboratory-based assays that are unsuited to clinical workflows.

This study aimed to test and validate a new nitrite point-of-care test (POCT) for oral medicine. Building on previous research, we developed a biosensing platform based on screen-printed carbon electrodes modified with a selective enzyme, the cytochrome *c* nitrite reductase. To adapt bioelectrodes for salivary analysis, they were further modified with a poly(vinyl) alcohol coating and a biochemical oxygen scavenger system (ascorbate oxidase and ascorbate). The nitrite biosensor achieved a suitable linear range of 5–300 μM, with a sensitivity of 0.015 μM^{-1}, validated against the gold-standard Griess method. Unlike the Griess reaction, the biosensor was unaffected by sample turbidity, rendering centrifugation unnecessary. Additionally, we observed that sample freezing altered nitrite concentrations, increasing levels in non-centrifuged samples, while decreasing them in previously clarified samples. These findings highlight the need for real-time analysis and call into question the reliability of previously published data that overlooked these variables. Therefore, our results demonstrate the potential of the nitrite biosensor as a novel salivary POCT and emphasize the critical need for standardized sample-handling protocols.

1. Introduction

Periodontal disease (PD) is a significant global health concern, affecting populations in both developed and developing countries [1]. According to the World Health Organization (WHO), oral diseases affect approximately 3.5 billion people worldwide, with PD accounting for 1 billion cases among adults. PD is an inflammatory condition affecting the structures surrounding the teeth, including the bone and gingival tissue. It is primarily caused by bacterial accumulation due to inadequate oral hygiene, malnutrition, and the use of certain medications [2]. The disease initially presents as gingivitis, characterized by redness, swelling, and potential bleeding of the gums. However, if left untreated, gingivitis may advance to periodontitis - a more severe form marked by gum recession, persistent bleeding, and eventual bone and tooth loss [3].

Modern therapeutics prioritize bacterial plaque control, and in severe cases, may involve removal of the gingival gums [4]. Although treatment can slow its progression, it may not fully halt the disease [5]. The classification of periodontitis is defined during the initial diagnostic appointment and once assigned, cannot regress to earlier, less severe stages of the disease [6]. To assess PD's severity (grade) and progression rate (stage), clinicians rely on standardized diagnostic methods including periodontal charts, radiographic analysis, and evaluation of symptoms and gingival tissue morphology (e.g., color, texture, and contour) [7,8]. Additionally, they examine the presence of bacterial plaque, probing depth, bleeding on probing, and recessions, among others [9,10]. Although early detection greatly improves treatment outcomes, diagnosis often occurs only after the disease has advanced to

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https://doi.org/10.1016/j.sbsr.2025.100860
Received 13 May 2025; Received in revised form 3 August 2025; Accepted 4 August 2025
Available online 5 August 2025

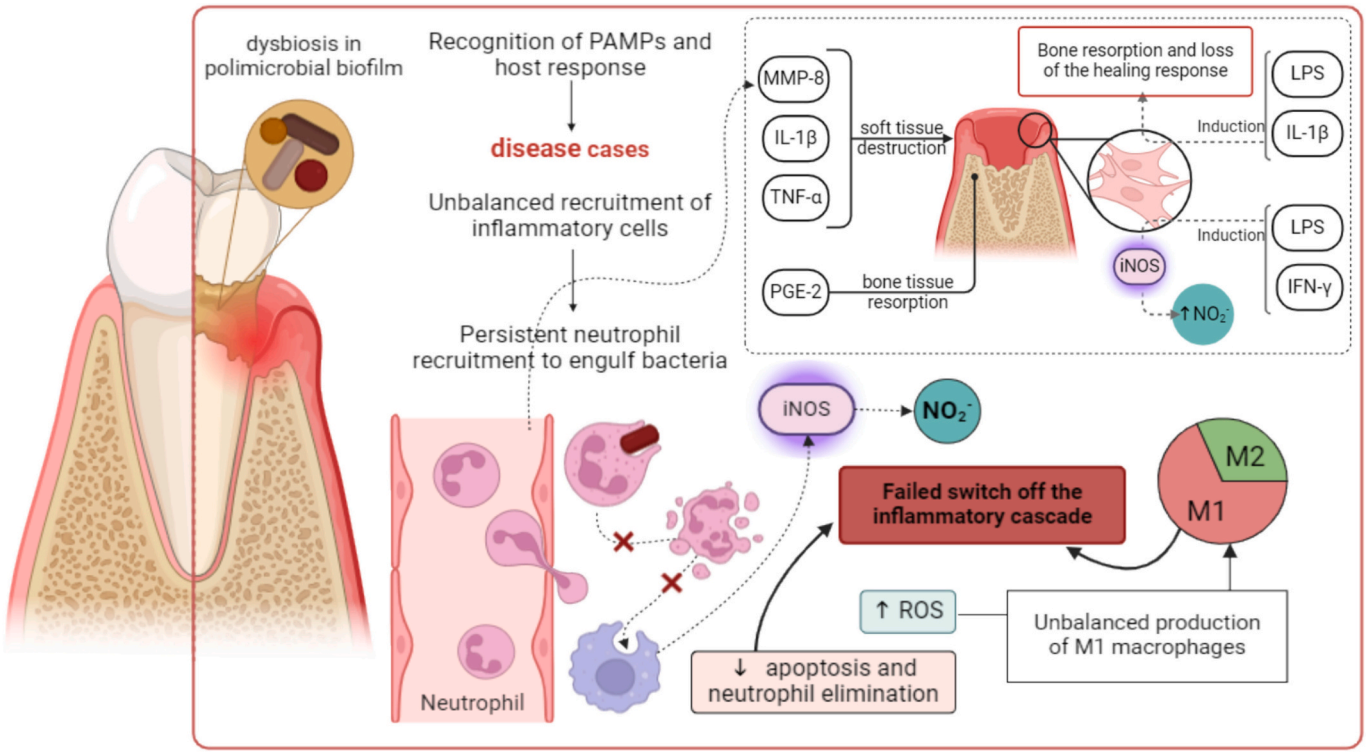

Fig. 1. The pathogenesis of periodontitis begins with the disruption of the homeostatic microbial biofilm. Dysbiosis in the polymicrobial biofilm impairs the recognition of pathogen-associated molecular patterns (PAMPs), leading to an inflammatory response. In periodontitis cases, there is an unbalanced recruitment of inflammatory cells, particularly neutrophils, which are responsible for bacterial phagocytosis. This dysregulated recruitment results in impaired neutrophil apoptosis. Additionally, the excessive accumulation of neutrophils inhibits macrophage-mediated digestion. During the immune response, increased ROS production blocks the macrophage transition from pro (M1) to anti-inflammatory (M2) phenotype, leading to a failure in resolving inflammation. Furthermore, immune cells release key biomolecules, such as MMP-8, IL-1β and TNF-α, which contribute to soft tissue destruction, and (PGE-2), which is associated with bone resorption. The inflammatory response also activates inducible nitric oxide synthase (iNOS), leading to the production of NO•, which is subsequently oxidized to nitrite. Fibroblasts also play a crucial role, as they are activated by molecules such as lipopolysaccharides (LPS), IL-1β, and interferon-gamma (IFN-γ). Depending on the specific stimulus, fibroblasts contribute to bone resorption, impaired bone healing, and the activation of iNOS (Adapted from [3,4,6]).

the periodontitis stage. Therefore, a rapid and specific diagnostic approach is essential for identifying the initial stages of the disease. The integration of conventional clinical methods with novel techniques for detecting specific biochemical markers of PD could offer significant benefits to both clinicians and patients. The pathogenesis of periodontitis is initiated by immune system activation in response to dysbiosis of pathogenic microorganisms within bacterial biofilms. This response triggers the release of pathogen-associated molecular patterns, which bind to host cells, initiating an inflammatory cascade. As a result, there is excessive recruitment of immune cells – such as phagocytes and apoptotic cells - to the affected site. A secondary defense line involves macrophages, which release inflammatory mediators, enzymes, and cytokines - including interleukin-1β, tumor necrosis factor-α, prostaglandin E2 and matrix metalloproteinases. Each of these molecules plays a critical role in the progression of the disease (Fig. 1) [9,11]. Among the enzymes involved, the inducible nitric oxide synthase (iNOS) produces nitric oxide (NO·), a reactive nitrogen species that disrupts the balance between macrophages' pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes [10].

Interestingly, the stable metabolite of nitric oxide – nitrite (NO_2^-) - has attracted attention as a potential biomarker for PD, as elevated inducible iNOS activity may lead to increased nitrite levels in the oral cavity. Several studies established a correlation between nitrite concentration and the severity and progression of PD (Table 1). While reversing periodontitis is not currently possible, developing efficient methods for the early detection of nitrite levels could significantly aid in disease management. However, approximately two-thirds of these publications reported elevated nitrite levels in saliva, gingival crevicular fluid (GCF), or both, in individuals with chronic periodontitis [12], whereas the remaining studies observed lower nitrite concentrations in the saliva of individuals with PD compared to healthy controls [13]. These conflicting findings hinder the establishment of a definitive correlation between periodontitis and nitrite concentration, highlighting the need for further research employing well-designed studies and more robust analytical methodologies.

Traditionally, nitrite is quantified using techniques such as ion chromatography, gas chromatography, UV–Vis spectrophotometry, fluorescence spectrophotometry, electrophoresis, and chemiluminescence, among others [14,15,16]. The most widely used method is the Griess method, a colorimetric assay first proposed by Johann Peter Griess in 1879, which is followed by spectrophotometric measurements [17]. The principle behind the method involves the reaction of nitrite under acidic conditions with sulfanilamide to form a diazonium salt, which then couples with N-(1-naphthyl)ethylenediamine dihydrochloride, producing a purple azo compound proportionally to the nitrite concentration [18,19,20]. Despite its widespread use, the Griess method has limitations. Colorimetric assays often require large, specialized equipment and high volumes of reagents and samples, and are therefore complex for routine nitrite analysis. Additionally, optical methods can be affected by matrix interferences, prolonged analysis times, and a lack of portability [14]. In clinical diagnostics, there is a growing need for quick tests that enable effective, straightforward, and real-time analysis in point-of-care settings. Biosensing technology addresses these needs by offering portable methodologies with stable and specific responses, facilitating on-site quantification. They also provide selectivity for the analyte and are generally unaffected by the sample matrix, making them

Table 1
Selected works on nitrite analysis in human saliva using the Griess method.

Ref.	Study Group	Sample	NO_2^- concentration (μM)
[29]	Moderate periodontitis	Unstimulated saliva	7.8
	Advanced periodontitis		15.8
	Healthy subjects		5.86
[30]	Periodontitis	Unstimulated	537.7
	Healthy subjects	saliva	241.0
[12]	Moderate periodontitis	Unstimulated saliva	16.5
	Advanced periodontitis		16.4
	Healthy subjects		5.7
[31]	Moderate periodontitis	Unstimulated saliva	6.4
	Advanced periodontitis		19.1
	Healthy subjects		n.d.
[32]	Periodontitis	Unstimulated	600
	Healthy subjects	saliva	300
	Periodontitis	Stimulated	700
	Healthy subjects	saliva	500
[33]	Periodontitis	Unstimulated	n.d. *
	Healthy subjects	saliva	Higher NO_2^- levels in periodontitis patients when compared with healthy subjects.
	Periodontitis	Stimulated	
	Healthy subjects	saliva	
[34]	Moderate Periodontitis	Unstimulated saliva	233.2
	Advanced periodontitis		524.9
	Healthy subjects		316.1
[35]	Periodontitis	Unstimulated	33.2
	Healthy subjects	saliva	173.7
[36]	Periodontitis	Unstimulated	5.6
	Healthy subjects	saliva	8.7
[37]	Periodontitis	Unstimulated	11.1
	Healthy subjects	saliva	22.4
[13]	Periodontitis	Stimulated	57.3
	Healthy subjects	saliva	92.5

* not defined (n.d.)

suitable for complex physiological specimens, such as saliva, where viscosity and solid residues may be an issue. Moreover, biosensors offer several advantages, including the elimination of sample pre-treatment, reagent addition, and the need for large sample volumes [21].

Our research group developed an enzyme-based nitrite biosensor that was proven to detect nitrite with high selectivity and sensitivity [22–24]. It integrates electrochemical transduction (via voltammetry or amperometry techniques) with the biorecognition element, cytochrome *c*-type nitrite reductase (NiR), which catalyzes the six-electron reduction of nitrite to ammonium (NH_4^+) when electrochemically activated at negative potentials (Fig. 2) [14]. Upon miniaturization of the electrochemical setup with disposable carbon screen-printed electrodes (SPEs), preliminary tests were conducted using complex physiological samples, such as urine and plasma, demonstrating the potential of this bioanalytical tool as an effective point-of-care test (POCT) [25,26].

In this study, we tested and statistically validated this novel enzymatic nitrite biosensor in saliva samples, using the gold standard Griess method as a reference. Because salivary nitrite concentrations are

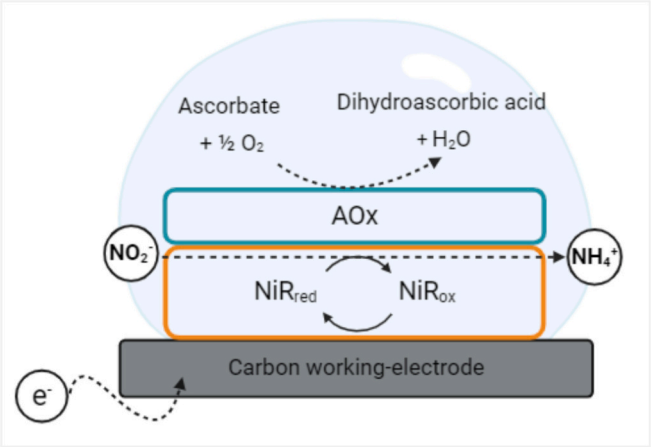

Fig. 2. Schematic model of the enzymatic nitrite biosensor with direct electrochemical transduction. The electrochemically reduced enzyme NiR, transfers six electrons from the electrode to nitrite (NO_2^-), forming the product ammonium. The top layer is composed of a scavenging method to remove oxygen by of molecular O_2 to H_2O; NiR – cytochrome *c* nitrite reductase; NiR_{red} – NiR reduced state; NiR_{ox} – NiR oxidized state; AOx – Ascorbate oxidase (adapted from [15]).

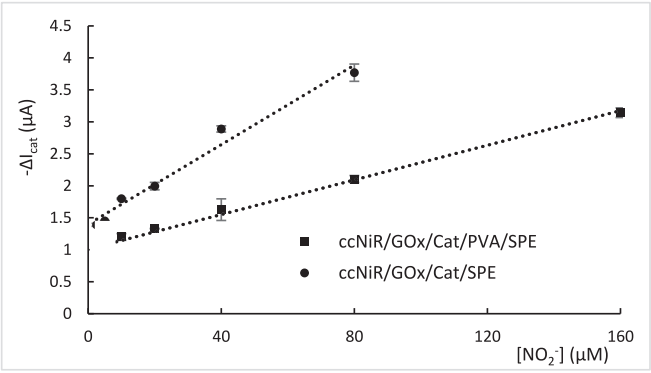

Fig. 3. Linear correlation between the ΔI_{cat} at −0.5 V. CVs were collected as a response to NO_2^- standard solutions (2.5–160 μM) with a scan rate of 20 mV s^{-1}. The supporting electrolyte was 0.1 M Tris-HCl buffer, pH 7.6, with 0.1 M KCl containing 80 mM glucose. Each data point is the average of duplicates. The data points were fitted to a straight line using the linear regression method, resulting in the following equations: $\Delta I_{cat} = 0.0311\ [NO_2^-] + 1.3991$, $r^2 = 0.978$ (● without PVA) and $\Delta I_{cat} = 0.0135\ [NO_2^-] + 1.009$, $r^2 = 0.993$ (■ with 1.25 % PVA).

typically high (Table 1), a precise fine-tuning of the biosensor performance was initially required. To enable accurate quantification of elevated nitrite levels, a polymeric coating of poly(vinyl alcohol) (PVA) was added to the sensing surface of the SPEs (test strips), making a diffusion barrier that increases the upper limit of detection [21]. To avoid the interference from dissolved oxygen, which is electrochemically active at the same potential range, the biosensor also included an alternative biochemical oxygen scavenging system consisting of a single enzyme - ascorbate oxidase (AOx) - which catalyzes the oxidation of *L*-ascorbate to dehydroascorbic acid while concurrently reducing dissolved oxygen (O_2) directly to water (Fig. 2). This approach mitigates the problems presented by the more commonly used glucose/catalase (GOx/Cat) bi-enzymatic system, specifically the risk of reactive oxygen species generation and interference from free flavin adenine dinucleotide (FAD), which produces a cathodic wave that overlaps with the nitrite electrocatalytic signal [27]. In this work, AOx and ascorbate were directly immobilized onto the electrode surface, as described in [28]. As

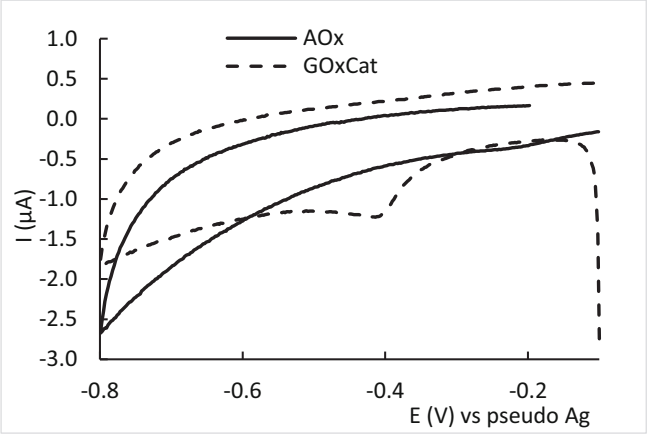

Fig. 4. Cyclic voltammograms (scan rate 20 mV s^{-1}) of AOx/SPE (filled line) and GOx/Cat/SPE (dashed line) modified SPEs, in the presence of their substrates, i.e., 100 mM ascorbate, and 80 mM glucose, after waiting 3 and 5 min, respectively. Supporting electrolyte: 0.1 M Tris-HCl buffer. pH 7.

for the potential interference from nitrates, it was not considered as a threat, as our previous works demonstrated that the biosensor response is not affected by this ion [22–24].

Furthermore, to identify potential sources of bias, we assessed the influence of sample pre-conditioning (freezing and centrifugation) on nitrite levels. Subsequently, under optimized experimental conditions, we applied the nitrite POCT in a pilot clinical observational study involving individuals with PD and periodontally healthy controls, aiming to explore potential correlations between salivary nitrite levels and periodontitis.

2. Materials and methods

2.1. Reagents and solutions

Sodium nitrite (≥ 99 %) was purchased from Normapur®. Trizma, Sodium hydroxide 0.02 % (≥ 98 %), Hydrochloric acid 37 %, Sulfanilamide (≥ 98 %), NED (≥ 98 %), Acetic acid (≥ 99 %), and poly(vinyl alcohol) (PVA) (MW 89,000-98,000, 99 % hydrolyzed), were obtained from Sigma-Aldrich®. Sodium phosphate monobasic and Potassium phosphate dibasic trihydrate were purchased from Sigma-Aldrich®. Sodium chloride and potassium chloride were purchased from Fluka®. All reagents used were of analytical grade. Solutions were prepared using deionized water 18 MΩ cm) obtained from the Millipore MilliQ purification system. Ascorbate oxidase (from *Curcubita* sp. 1000–3000 units mg^{-1}) and *L*-ascorbate were purchased from Sigma-Aldrich®. The enzyme solutions were prepared in a phosphate buffer (composed of sodium phosphate monobasic and potassium phosphate dibasic), whereas *L*-ascorbate was dissolved in 0.1 M Tris-HCl, 0.1 M KCl, pH 7.6. The enzyme NiR (300 U mg^{-1}) from *D. desulfuricans* ATCC 27774 cells was purified in-house as described by Almeida et al. [38].

2.2. Participants recruitment

This study received approval from the Egas Moniz Ethics Committee, conducted in the Egas Moniz Dental Clinic. Participants were selected based on predefined inclusion and exclusion criteria. Individuals were eligible for inclusion if they were over 18 years of age and had at least 10 teeth. Exclusion criteria comprised pregnancy, patients suffering from autoimmune diseases, current or chronic use of xerostomizing medications, ongoing radiotherapy, history of periodontal treatment, and patients who have or have had respiratory disease. All participants were fully informed about the study's objectives and the anonymity of their participation and were asked to sign an informed consent form. The data collected was used exclusively for statistical analysis. Prior authorization was obtained from the Egas Moniz Dental Clinic to access patient data. In the first phase, we examined the influence of pre-treatment procedures – specifically, centrifugation and freezing – on saliva samples, using both the biosensor and Griess methods to quantify nitrite levels. A total of 44 saliva samples were collected for this purpose.

2.3. Saliva sampling

Participants first rinsed their mouths with water, and then provided saliva samples via passive drooling, without any stimulation. Each sample was immediately placed on ice for no longer than 10 min and then divided into two groups. One group was analyzed immediately to eliminate storage time as a variable (F0), while the other was aliquoted and stored at −20 °C to assess the effect of freezing (F1); here, "F" denotes freezing. In parallel, to evaluate the effect of turbidity, each sample was further divided: one subgroup was analyzed without clarification (C0), and the other was centrifuged at 10,000 *g* for 10 min (C1), with "C" indicating centrifugation. This design resulted in four experimental groups based on the combination of both variables: C0F0, C0F1, C1F0, and C1F1.

A total of 63 samples were initially collected. However, based on specific inclusion and exclusion criteria, some samples were excluded from the analysis, making the final number of samples used for statistical evaluation variable across different analyses. For instance, following saliva collection, patient data were verified, and some results were excluded due to insufficient clinical information. Additionally, samples were excluded if the nitrite concentration was below the detection limit or if the sample volume was inadequate.

2.4. Biosensor

SPEs model DS-110, consisting of a carbon working electrode, a carbon counter electrode, and a silver (Ag) pseudo-reference electrode, were sourced from DropSens®. To modify the SPEs, a layer of NiR (1 mg mL^{-1}) was drop-casted onto the working electrode and dried in an oven at 40 °C, for 30 min. Subsequently, the biochemical oxygen scavenging system was applied over the initial layer, coating it with AOx (1000–3000 U mg^{-1}) and then drying it in an oven at 30 °C, for 10 min [27,28]. Finally, 1.25 % PVA solution was added to cover the enzyme layers, allowing the PVA to dry at room temperature for 30 min. The biosensors were used immediately after preparation or stored in thermosealable pouches obtained from Amcor Flexible, at 4 °C.

2.5. Electrochemical measurements

The electrochemical measurements were conducted using two techniques: cyclic voltammetry (CV) and chronoamperometry (CA). These assays were performed with a *Sensit Smart* portable potentiostat from PalmSens®, operated via PSTouch software (PalmSens®) for control and data acquisition. Cyclic voltammograms were recorded at room temperature (21 ± 2 °C) within a potential range of 0.0 V to −0.6 V at a scan rate of 20 mV s^{-1}. Catalytical currents (ΔI_{cat}) were determined at the cathodic peak and subtracted from the correspondent current in a blank assay (without nitrite). For chronoamperometry, a fixed potential of −0.5 V (vs. Ag pseudoreference) was applied, with ΔI_{cat} measured after 60 s. The biosensors were regularly calibrated over a concentration range of 0–250 μM, with results fitted to a linear model using linear regression. The nitrite quantification was performed using 20 μL of salivary fluid, diluted in an electrolyte buffer composed of 0.1 M Tris-HCl with KCl adjusted to pH 7.6, and 100-mM ascorbate solution. A 50 μL drop of the diluted sample was placed onto the working electrode, ensuring coverage of all electrodes. After 3–4 min, oxygen was removed, and the measurement was initiated.

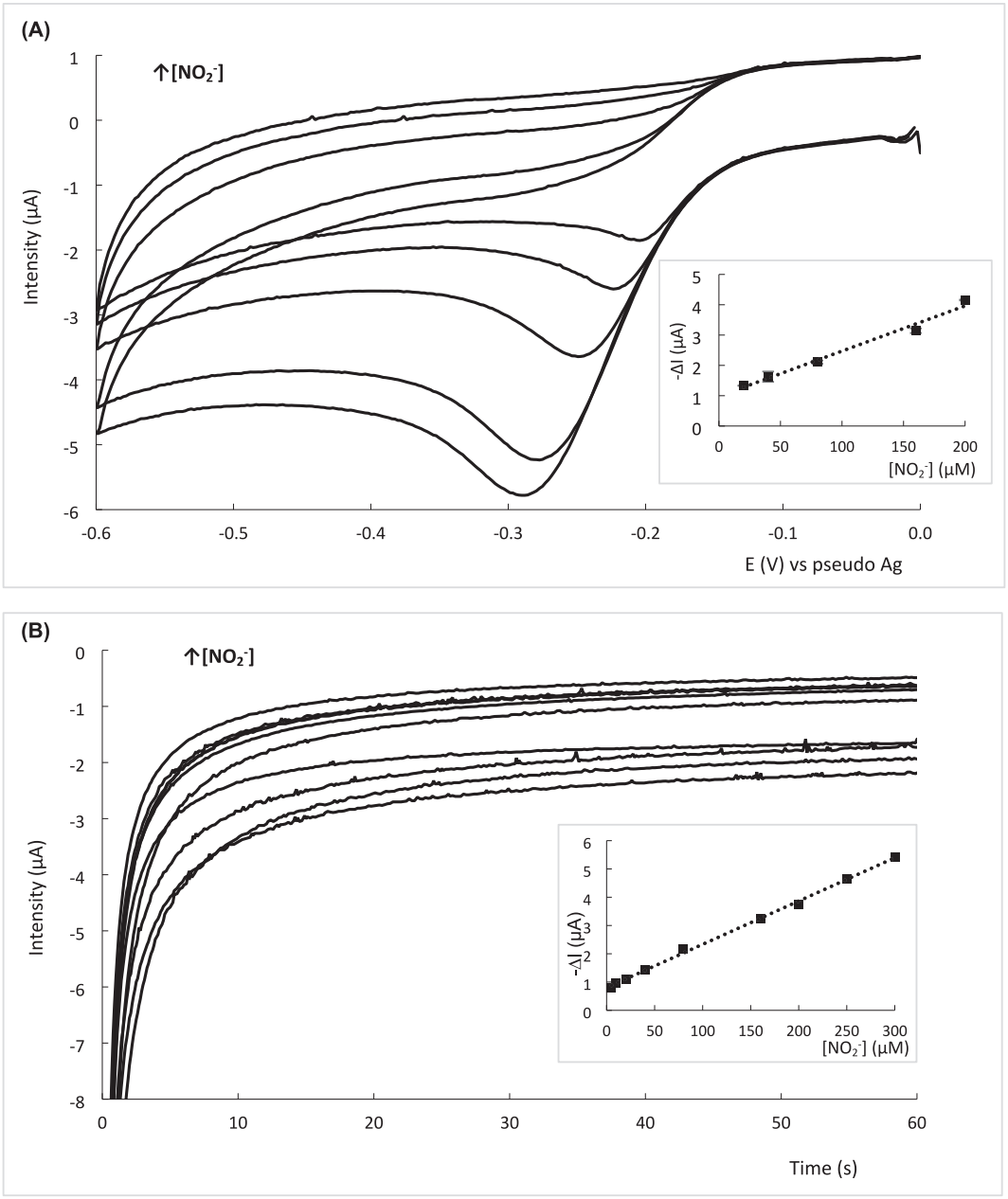

Fig. 5. **(A)** Cyclic voltammograms (20 mV s^{-1} scan rate) of supporting electrolyte (0.1 M Tris-HCl buffer, pH 7.6, 0.1 M KCl) containing 100 mM ascorbate and nitrite (linear range, 20–200 μM). Measurements were recorded with NiR/AOx/PVA/SPE, and the ΔI_{cat} was measured at the cathodic peak height. Nitrite calibration curve using the CV electrochemical technique. Each data point is the average of duplicates. The data were fitted to a straight line using the linear regression method, resulting in the following equation: $\Delta I_{cat} = 0.014\ [NO_2^-] + 0.981$, $r^2 = 0.982$, and a linear range of 20 to 200 μM. **(B)** Amperograms at −0.5 V of supporting electrolyte (0.1 M Tris-HCl buffer, pH 7.6, with 0.1 M KCl) containing 100 mM ascorbate and nitrite (linear range of 2.5–300 μM). Measurements were recorded with NiR/AOx/PVA/SPE, and the ΔI_{cat} was measured after 60 s. Nitrite calibration curve using the CA technique. Each data point is the average of duplicate assays. Data were fitted to a straight line using the linear regression method, delivering the following equation: $\Delta I_{cat} = 0.015\ [NO_2^-] + 0.802$, $r^2 = 0.998$, and a linear range of 5 to 300 μM.

2.6. Griess method

For this method, stock solutions of 1 % m/V sulfanilamide (SA) and 0.02 % m/V N-(1-naphthyl)ethylenediamine dihydrochloride (NED) were prepared. To construct the calibration curve, nitrite standards, deionized water, SA, and NED were sequentially added to a plastic cuvette. After a reaction time of 10 min, the absorbance was measured at 540 nm using a THORLabs® portable spectrophotometer or a Thermo Scientific® Helios Omega UV–Vis bench spectrophotometer. The same protocol was employed to quantify nitrite in real samples, with nitrite standards substituted by saliva.

2.7. HPLC-ionic chromatography

Nitrite analyses were executed in duplicates using an ion chromatography system model ICS3000 (Dionex, Sunnyvale, CA, USA) equipped with a conductivity detector. The data were processed with the software Chromeleon 6.8 (Dionex, Sunnyvale, CA, USA). In all analyses, an IonPac AS11-HC column (4 mm I.D. × 250 mm) with a guard column (4 mm × 50 mm) was used (Thermo Scientific, Waltham, Massachusetts, USA). Analyses were run at 25 °C, under isocratic conditions, with a mobile phase composed of 5 mM NaOH, at a flow rate of 1 mL/min.

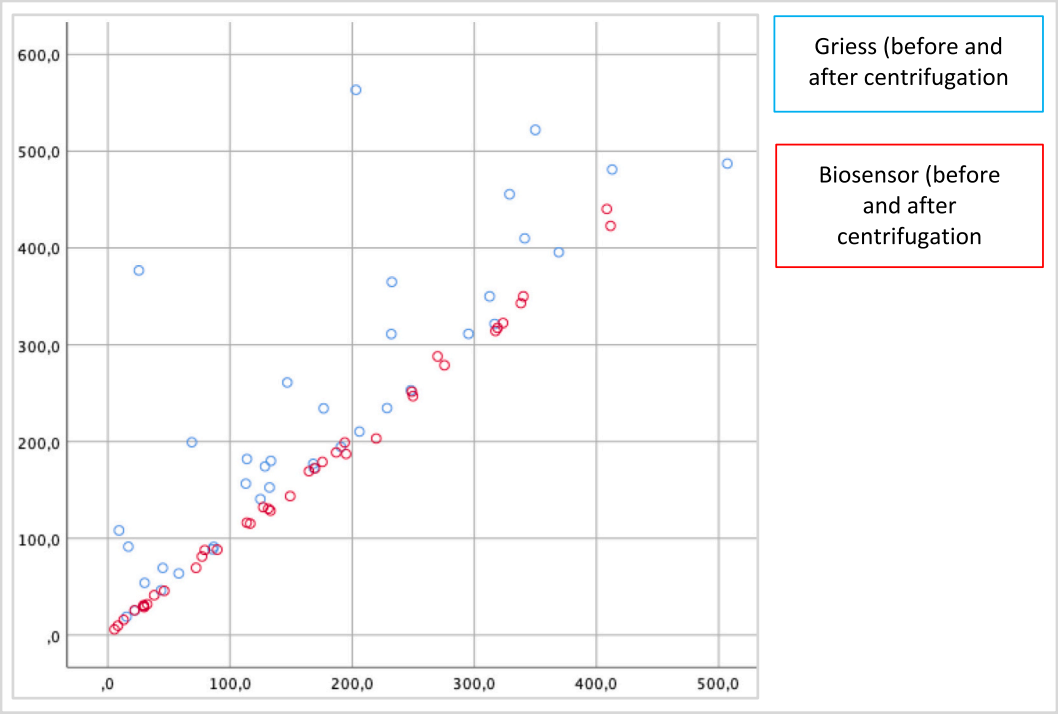

Fig. 6. Influence of centrifugation on nitrite quantification with the biosensor (red circles) and Griess (blue circles) methods. Dispersion plot of nitrite concentration in saliva samples (freshly collected) before (C0F0; y-axis), and after (C1F0; x-axis) centrifugation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.8. Statistical analysis

All data were recorded in Microsoft Office Excel® and analyzed using IBM SPSS Statistic 27.0 software. Descriptive and inferential statistical analyses were performed, with a significance level of 5 % applied to all inferential tests.

3. Results and discussion

3.1. Nitrite quantification

3.1.1. Optimization of biosensor response

Developing a new enzymatic biosensor requires addressing several factors that influence its electrochemical response. Therefore, before clinical testing, we identified and tackled key issues to enhance the nitrite biosensor's performance. To optimize response and minimize errors, we examined the influence of an additional polymer coating, oxygen scavenger system, and the electrochemical technique used. First, we fixed the biochemical oxygen scavenging system using the previously established GOx/Cat/glucose combination and tested the effect of a 1.25 % PVA coating to protect the enzyme layer, employing cyclic voltammetry exclusively. This last step aimed to enable the detection of higher nitrite concentrations, as the polymer film provides further protection and acts as a diffusion barrier for the analyte [21]. Our findings indicate significant differences in performance between biosensors with and without PVA (Fig. 3). The polymer reduced the sensitivity from 0.0311 to 0.0135 μM^{-1} and increased the lower quantifiable concentration (from 2.5 to 10 μM) but doubled the upper detection limit (from 80 to 160 μM) as required, making it more suitable for detecting the nitrite concentrations usually found in saliva specimens [10,32,36]. Furthermore, the correlation coefficient was much better when electrodes were coated with PVA. Therefore, all subsequent measurements were performed using test strips coated with a 1.25 % PVA layer.

Nitrite detection using reductase enzymes is challenging due to the negative potential required to drive the electrochemical reaction, as the cathodic reduction of dissolved oxygen (O_2) into hydrogen peroxide (H_2O_2) masks the electrocatalytic signal. Initially, our group used the bienzymatic oxygen scavenging system glucose oxidase GOx/Cat mixture (1:1 ratio) with glucose as the substrate [26,27]. Subsequently, we tested a novel biochemical system composed of a single enzyme, ascorbate oxidase (AOx), with ascorbate as substrate. In this system, AOx catalyzes the direct reduction of the co-substrate oxygen into water, with no intermediate ROS formation, whereas in the GOx/Cat system, GOx converts O_2 to H_2O_2, which is further reduced to water by catalase [27]. Fig. 4 shows the CVs obtained with the two oxygen scavenging systems, after incubation with their respective enzyme substrates. These results demonstrate that the alternative AOx/ascorbate system removes oxygen effectively, delivering a flat baseline. In contrast, the GOx/Cat/glucose system shows a small cathodic wave near −400 mV vs. the Ag pseudo-reference, potentially overlapping with the peaks developed in the presence of low nitrite concentrations (Fig. 4).

To avoid this interference and, on the other hand, eliminate the risk of H_2O_2 release, which can damage an enzyme-based biosensor like ours, we opted to replace the GOx/Cat/glucose system with the new AOx/ascorbate approach, ensuring a clearer nitrite electrocatalytic signal.

Finally, we also tested two dynamic electrochemical methods – CV and CA - for nitrite analysis (Fig. 5A and B, respectively).

These techniques differ in their analytical approaches: for CV, the catalytic current (ΔI_{cat}) is calculated at the cathodic peak, whereas in CA, ΔI_{cat} is measured after 60 s, with the background current subtracted. Upon evaluating the analytical performance of the biosensor with the NiR/AOx/PVA/SPE configuration, we observed a linear range of 20–200 μM for CV, and 5–300 μM for CA. The sensitivity was similar for both methods: 0.014 μM^{-1} and 0.015 μM^{-1} for CV and CA, respectively, but the latter provided a broader linear range. Additionally, CA is faster and easier to analyze, making it a suitable choice for clinical testing. Nevertheless, voltammetric analysis remains valuable during early biosensor development stages, as it provides insights into ongoing electrochemical processes and potential interferences. It should be noted

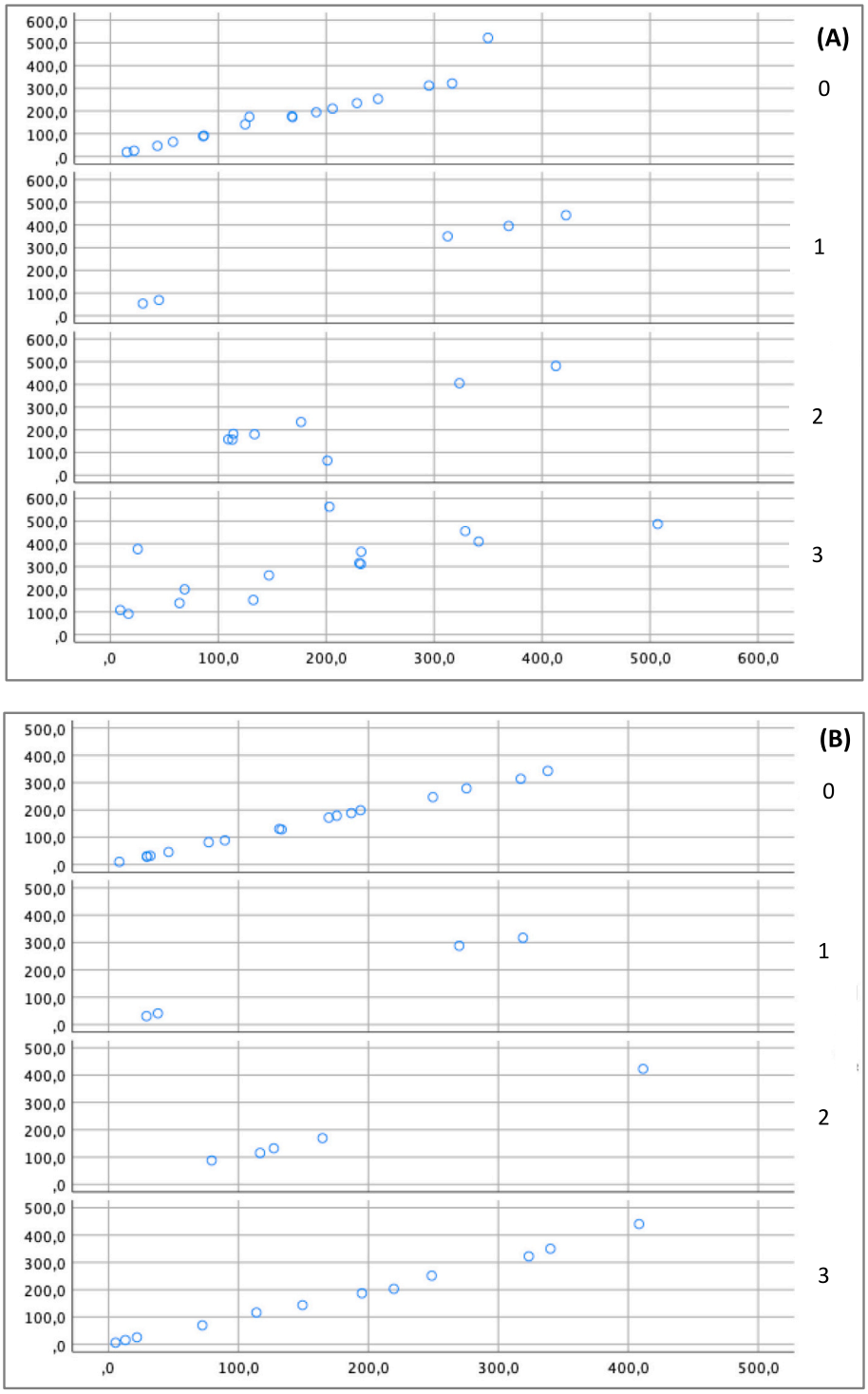

Fig. 7. Dispersion plot of nitrite concentration measured using **(A)** the Griess method ($N = 44$), and **(B)** the biosensor ($N = 38$), according to turbidity levels: 0 – clear samples, 1 – low turbid samples, 2 – turbid samples, 3 – very turbid samples.

that other analytical parameters such as selectivity, reproducibility, and stability, were not addressed in this work, as they have been thoroughly reported in previous publications [25,26].

3.1.2. *Griess method*

Nitrite concentrations were also measured using the Griess reaction colorimetric method to compare and validate the biosensor results against this gold standard approach. This optical assay provided a range from 2.5 to 25 μM with a sensitivity of 0.965 μM^{-1} (see results in the Supplementary Material – Fig. S1). One should emphasize that due to the limited detection range, an additional sample dilution step was required. Additionally, this method involves large-scale equipment and demands mixing the sample with two reagents, followed by a 10 min incubation period for color development, before measurements, which makes it less suitable for clinical settings.

Table 2
Freezing effect on centrifuged and non-centrifuged saliva samples.

Centrifugation before freezing samples	N	Number of samples with NO_2^- variation above the error	
		Griess	Biosensor
Yes (C1F0 vs C1F1)	14	12 ↓ *	12 ↓ *
No (C0F0 vs C0F1)	14	12 ↑ **	12 ↑ **

* 10 to 60 % decrease in nitrite concentration.
** 10 to 50 % increase in nitrite concentration.

3.2. Sample pretreatment

The evaluation of saliva pretreatment on nitrite quantification was conducted to study the interference of sample turbidity with the Griess spectrophotometric method and monitor changes in nitrite levels during cold storage. Typically, turbid samples such as saliva are cleared of suspended particles through centrifugation. As the colorimetric assay is normally conducted using a bench spectrophotometer in central laboratories, samples are often frozen prior to analysis. Therefore, to reproduce conventional protocols, in this study, samples were divided into smaller aliquots as follows: i) as freshly collected without any pretreatment step (C0F0); ii) as freshly collected and immediately centrifuged (C1F0); iii) stored at −20 °C, without previous centrifugation (C0F1); and iv) stored at −20 °C, after centrifugation (C1F1). They were all tested for nitrite using both the Griess and biosensor methods.

3.2.1. Centrifugation

To assess the specific effect of centrifugation, two groups of saliva samples were initially pooled as described in Section 3.2; one group was centrifuged and the other not (C1F0 and C0F0, respectively), and none of them was freeze-stored. All samples were analyzed in parallel using both the biosensor ($N = 38$, including healthy and PD individuals) and the Griess method ($N = 44$, including healthy and PD individuals), and the results were statistically compared (Fig. 6).

Our findings indicate that the biosensor exhibits a linear correlation between the two groups, demonstrating that results are unaffected by sample turbidity, which means that samples do not require a pre-centrifugation step to achieve accurate measurements. In contrast, data obtained from the Griess method were affected by sample turbidity, resulting in significant differences in nitrite concentration between pre- and post-centrifuged samples.

To better assess the impact of turbidity on the results, saliva samples were scored for opacity on a scale from 0 to 3, with 0 representing a clear sample and 3 indicating a highly turbid one. As shown in Fig. 7A, turbidity significantly affected the accuracy of the Griess method, with the most pronounced discrepancies observed in samples assigned a turbidity score of 3 (Fig. 7A and B). Due to its high content of cells and food particles, saliva scatters light, leading to increased absorbance and an overestimation of nitrite concentration [19]. Moreover, this process is time-consuming and requires access to a refrigerated centrifuge.

3.2.2. Freezing effect

A smaller group of saliva samples ($N = 14$) was re-analyzed to investigate the influence of 10-day cold storage at −20 °C on nitrite concentration. These samples were initially examined within 10 min of collection using both the Griess and biosensor methods, with and without centrifugation; afterward, samples were aliquoted and frozen at −20 °C, rendering the four subgroups described in 3.2, i.e., freshly collected with (C1F0) and without (C0F0) centrifugation, frozen with (C1F1), and without centrifugation (C0F1). Table 2 shows the output of the qualitative comparison of results between subgroups C0F0 vs C0F1 (without centrifugation) and C1F0 vs C1F1 (with centrifugation). Overall, post-freezing analyses showed a 10 to 60 % decrease in the nitrite content in centrifuged samples, while non-centrifuged samples showed a 10 to 50 % increase in nitrite concentration, regardless of the analytical technique. The observed reduction in nitrite levels in centrifuged saliva suggests this ion is unstable in frozen conditions, likely due to its progressive oxidation into nitrate. However, the observed increase in nitrite concentration in non-centrifuged samples after freeze-storage is more challenging to explain. One possible explanation is the activity of nitrate reductase enzymes from commensal bacteria naturally present in the oral cavity. If nitrate residues are present in saliva, this bacterial activity may contribute to the elevated nitrite level before being halted

Table 3
Nitrite content in two saliva samples, quantified by the Griess, biosensor, and HPLC techniques.

Samples	NO_2^- concentration (μM)		
	Griess	Biosensor	HPLC
S28	82 ± 3	81 ± 3	75.7 ± 0.5
S50	56 ± 2	53 ± 2	46

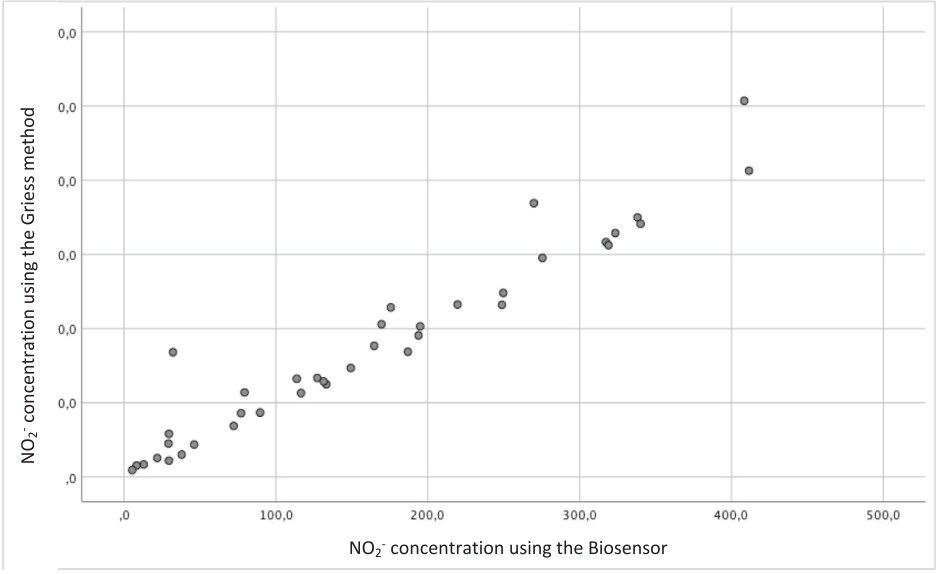

Fig. 8. Biosensor validation. Dispersion plot of nitrite concentration measured in saliva samples using the Biosensor and the Griess method, with $r^2 = 0.967$.

by sample freezing. Future studies should further investigate this hypothesis by evaluating both nitrate concentrations and the composition of the oral microflora.

3.3. Biosensor validation

The analytical performance of the biosensor ($N = 38$, including healthy and PD individuals) was validated by comparing its results with those obtained using the Griess method. To ensure consistency, only centrifuged saliva specimens were used for the Griess assay, as initial studies showed that optical methods are affected by sample turbidity. The statistical comparison revealed no significant differences ($p < 0.05$) between the results obtained by the two techniques (Fig. 8), thereby validating the biosensor methodology for measuring nitrite in human saliva without any sample preparation. This result is a critical step toward adapting the methodology for clinical application, where it could assist clinicians in disease diagnosis and facilitate future research.

Additionally, two saliva samples (previously centrifuged and frozen) were selected for nitrite analysis using ionic high-performance liquid chromatography (HPLC-ionic exchange), known for its high sensitivity in complex matrices. The results (Table 3) confirm the accuracy of the nitrite biosensor, as the nitrite concentrations measured were consistent with those obtained via the Griess and HPLC analysis.

Our findings indicate that the biosensor's response to nitrite is reliable and unaffected by saliva turbidity, highlighting its potential for use in real-time point-of-care testing. Importantly, it is simple to use and much quicker (each sample takes less than 5 min) than lab-based methods.

4. Conclusions

In this work, we employed for the first time a nitrite electrochemical biosensor, previously developed and characterized by our research group, in saliva specimens [25,27]. Additional electrode modifications introduced herein enabled the biosensor to operate in the open air, providing a broad linear range within typical salivary nitrite. The biosensor's performance was successfully validated against the conventional Griess method, proving to be accurate, rapid, and easy to use. This enzyme-based electrochemical biosensor is thus, a good candidate to serve as nitrite POCT and replace conventional bench methods in clinical studies.

The study also assessed the impact of pre-analytical procedures – centrifugation and freezing – on both the biosensor and the colorimetric Griess method. Our results demonstrated that centrifugation is not mandatory for the biosensor, as sample turbidity did not affect its performance, addressing key limitations of conventional colorimetric assays. Freezing, on the other hand, significantly altered salivary nitrite levels regardless of the analytical method used, underscoring the necessity of real-time analysis. Furthermore, our findings demonstrated that freezing samples without prior clarification can impact nitrite concentrations - an aspect previously overlooked, which may have influenced the nitrite measurements reported in earlier studies. Therefore, to ensure analytical accuracy, saliva samples should be analyzed immediately after collection. These findings emphasize the critical importance of using standardized sample-handling protocols in saliva biomarker research.

To conclude, this study demonstrates the potential of the new nitrite POCT in the accurate quantification of salivary nitrite, which should be assessed in real-time, during dental consultation. These important outcomes should be considered in the design and execution of future clinical studies on the potential role of nitrite as a PD marker.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbsr.2025.100860.

CRediT authorship contribution statement

Sara Rodrigues Gaspar: Writing – original draft, Methodology, Investigation, Formal analysis. **Luís Proença:** Formal analysis. **Ricardo Alves:** Supervision, Methodology. **Maria Gabriela Almeida:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Maria Gabriela Almeida has patent #P4377684A4·2024 | US20240167071A1 pending to Maria Gabriela Almeida. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work is financed by national funds through the FCT – Foundation for Science and Technology, I.P., under the projects UIDB/04585/2020 (Research Center - CiiEM), UIDP/04378/2020 and UIDB/04378/2020 (Research Unit on Applied Molecular Biosciences - UCIBIO), and LA/P/0140/2020 (Associate Laboratory Institute for Health and Bioeconomy - i4HB), and for the research project 2022.04940.PTDC. The authors acknowledge Nuno Costa from Laboratório de Análises/REQUIMTE/LAQV at NOVA FCT, Portugal, for the acquisition of the ion chromatography data.

Data availability

Data will be made available on request.

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