Simple Melatonin Determination using disposable and low-cost lab-made Screen-Printed Carbon Electrode

*Ava Gevaerd[1,2]; Emily Y. Watanabe[1]; Bruno C. Janegitz[3], Márcio F. Bergamini[1]; Luiz H. Marcolino-Junior[1]**

[1]Laboratório de Sensores Eletroquímicos (LabSensE) – Departamento de Química, Universidade Federal do Paraná (UFPR), CEP 81531-980, Curitiba, PR, Brazil.

[2]Hilab, Rua José Altair Possebom, 800, CEP 81270-185, Curitiba, PR, Brazil.

[3]Laboratory of Sensors, Nanomedicine, and Nanostructured Materials (LSNano), Federal University of São Carlos, 13600-970, Araras, São Paulo, Brazil

Corresponding Author*: Luiz H. Marcolino-Jr (luiz1berto@ufpr.br)

Abstract

This work describes the preparation and application of a screen-printed electrode (SPE) for direct melatonin (MT) determination in samples of saliva and urine. The low-cost SPE was obtained using a simple and feasible 2D printing methodology and a lab-made conductive ink based on graphite powder and colorless nail polish. The proposed electrode showed similar electrochemical performance compared to a commercial SPE towards melatonin oxidation. Based on electrooxidation of melatonin recorded at +0.62V an electroanalytical method was developed using the lab-made SPE under square wave voltammetric conditions. Repeatability and reproducibility studies showed that the relative peak current values did not show significant differences between them. Under optimized parameters inherent to the methodology and voltammetric technique, the proposed SPE presented a linear dynamic range (LDR) between 0.25 to 75.0 μmol L^{-1} (R2 = 0.99), and calculated LOD and LOQ of 25.8 and 83.3 nmol L^{-1}, respectively, were obtained. The effect of other biologically relevant compounds such as glucose, urea, and estradiol was investigated, and non-significative signal interference was observed. The method proposed was successfully applied to MT determination in spiked samples providing good recovery values between 95.6 to 104%.

Keywords: Biomarker; Miniaturized System; Screen-printed Electrodes; Rapid Test.

1. INTRODUCTION

Melatonin (MT), also known as "sleep hormone," is produced in several tissues and biological cells, but mainly secreted by the brain through the pineal gland from tryptophan, and is classically associated with its night release, where its effects on circadian entrainment were recognized long ago. Its reference values vary according to the day, being lower in the morning, as its release occurs in the evening, as a way to "prepare" the body for rest[1]. Besides, these levels are age-dependent, and as age advances, MT levels in the body become lower [2]. As much as it is mostly sleep-related, MT modulates cellular function across a series of processes, and it is considered a powerful antioxidant and anti-inflammatory species, that mediates many of its effects by optimizing mitochondrial function and which act to dampen down inflammatory [3]. So, MT could be strongly associated with mitochondrial function and, therefore, with the regulation of the immune system.

In addition to having several other biological and therapeutic benefits [4–6], MT also has antiviral properties, and acting as an antioxidant and anti-inflammatory agent it can play against some comorbidities caused by viral and bacterial infections, such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[7], since, some studies indicate that people with MT low levels could be more affected by the most common side effects presented by viral infections. Because it can restrict viral infections, with the management of oxidative stress and inflammatory responses, as well as the regulation of immune responses, the use of this species can be fundamental in combating several respiratory infections by viruses[7], such as Influenza or even SARS-CoV-2.

The Covid-19 pandemic further emphasized the need for research and development of simple, rapid, and decentralized tests, which can assist in the early treatment of several diseases. Thus, the monitoring of key species, such as MT, which

can be used in different strategies, whether to treat or control the progress and symptoms of different diseases, in different biological fluids, is of great importance. In this context, this work described the simple and direct determination of MT in urine and saliva samples using a low-cost and lab-made screen-printed electrode (SPE) based on graphite and nail polish, using square wave voltammetry. Methodologies described for the determination of melatonin can be found in the literature [8–14]. Despite presenting a good analytical performance, with low LOD values and high sensitivities, many of them are based on modified devices that require time to be built, either to obtain the modifier or to build the sensitive platform, or devices that require pretreatment before each use. These extra steps end up making the methodologies time-consuming and difficult to apply in routine analysis, for example.

Electrochemical methods are a powerful tool for monitoring species of interest, especially in the health and pharmaceutical field. It is used as an alternative or complementary technique to conventional or separation techniques especially due to some advantageous characteristics such as high sensitivity, quick analysis, low cost, and ease of system miniaturization. They are devices that allow data collection and obtaining information with minimal reagents and sample manipulation, in real-time and in situ, and the results obtained can be analyzed and correlated with other parameters of the studied system [15–17].

So, the growing demand for more practical and straightforward analytical methods has driven the development of several types of electrochemical sensors, with special attention to miniaturized devices [18]. Despite their great versatility and high performance, most of them need to be renewed for reuse, since are expensive to be discardable [19]. The search for disposable devices with low-cost production and good accuracy is desirable in electroanalysis [20]. In this way, previous works have been shown

SPEs are suitable satisfactorily for this type of analysis, proving to be a viable alternative for quick measurements using disposable electrodes [21–24].

The development of SPEs [25,26] has offered a system designed with great simplicity and economy. It can be characterized by a single electrode array device, with working, auxiliary, and reference electrodes printed on the same support. The great versatility of these printed electrodes served as an incentive in the search for new construction possibilities. These possibilities involve the use of different substrates [27–29], different compositions of conductive ink [30–32], and different electrode arrangements [33] that add to these devices' characteristics of low-cost, accessibility, and applicability in several areas [34,35].

2. EXPERIMENTAL

2.1. Materials and Reagents

The chemicals were of analytical grade and used without previous purification. Graphite powder, Melatonin (MT) were purchased from Sigma-Aldrich®. Ethyl Acetate from Prudenquímica® (Presidente Prudente, SP, Brazil). Colorless nail polish from Risqué® (São Paulo, Brazil). Sodium phosphate dibasic anhydrous and phosphoric acid were purchased from Carlo Erba Reagents (Val de Reuil – France), and sodium phosphate monobasic monohydrate from Synth (Diadema, SP, Brazil) then used to prepare the phosphate-buffered solution (0.10 mol L^{-1}). The solutions were prepared with water purified using a Milli-Q system manufactured by Millipore (Bedford, MA, USA). An MT stock solution of 1.0 mmol L^{-1} dissolved in methanol was prepared, and less concentrated solutions were prepared by dilution. Phosphate buffer solution studied in the pH range from 2.0 to 7.0 was used as a supporting electrolyte.

2.2. Home Made Ink (HMI) and Electrode Printing

The SPE used was obtained as previously described [36]. Briefly, the conductive ink was made by a mixture of graphite, colorless nail polish, in proportions of 70:30 (% w/w), respectively, and ethyl acetate following a ratio of 100 μL of solvent for each 100 mg of graphite/nail polish, and mixed until complete homogenization. All the consumables for the SPE construction were purchased in the local market, Curitiba - PR, Brazil.

For the 2D printing, first, the polypropylene sheet (210 × 297 mm); 0.30 mm thickness), used as the substrate, was physically treated with sandpaper and cleaned with isopropyl alcohol to eliminate any remaining dust. After, a vinyl adhesive mask was fixed on the substrate to define the electrode outline, and the conductive ink previously

prepared is poured and spread with the aid of a spatula. Before it was all dried, the mask was removed to avoid cracks, and after drying, the electrode area is isolated using a piece of adhesive magic tape (3M®), and a silver-silver chloride paste layer (product code: C2030812P3; Gwent Electronic Materials Ltd, UK) is manually deposited on the reference electrode area. The drawing of the electrodes was made in AutoCAD® 2019 free student, exported to the software Silhouette Studio®, which was used by the Silhouette Cameo 3® cutting machine. The final SPE, as well as the configuration of the electrochemical cell, are represented in Figure 1A-C. The final working electrode area was 0.07 cm^2. Comparative studies were carried out using commercial SPE (ET090-40 Kanichi Carbon SPE, eDAQ, Inc.) which consists of a graphite working electrode, a graphite counter electrode, and an Ag/AgCl reference electrode.

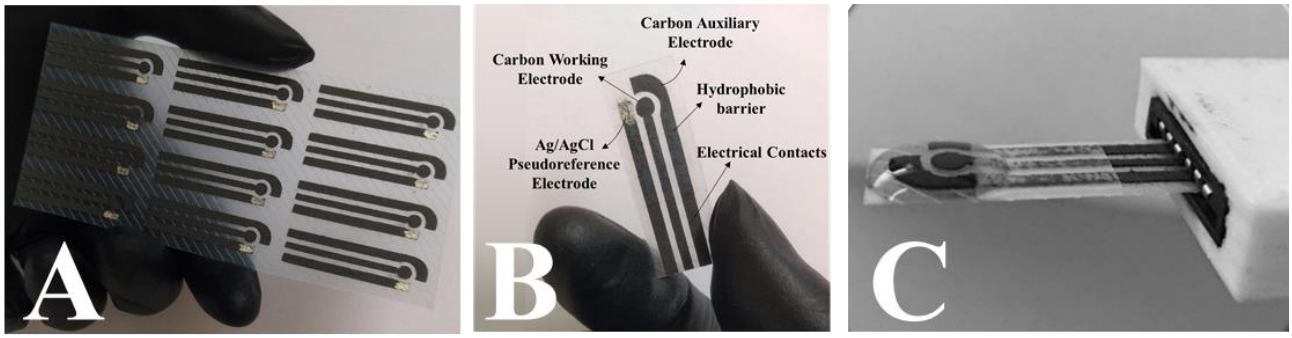

Figure 1. A) Final set of Screen-Printed Electrodes (SPE); B) Individual SPE and electrode configuration; C) Electrochemical cell read-to-use.

2.3. Electrochemical Measurements and Analytical Application

Electrochemical measurements were carried out using potentiostat/galvanostat µAutolab Type III (EcoChemie - Metrohm Autolab B.V., Netherlands). Aliquots of 100 µL of phosphate buffer solutions for blank and MT solutions with different concentrations were added directly to the SPE surface using a micropipette (Fig 1C). Cyclic (CV) and Square Wave (SWV) Voltammetry were used in a potential range of 0.2 to 1.0 V. The

baselines were adjusted using the moving average tool available in the NOVA 2.1 software. Electrochemical Impedance Spectroscopy (EIS) measurements were performed using Potentiostat/ Galvanostat PGSTAT204 (Metrohm Autolab B.V., Netherlands) managed by FRA32 module using a potential peak of melatonin, in a frequency range of 100 kHz to 1 mHz, and amplitude of 10 mV.

Analytical validation of the proposed method was performed by using the SPE for the determination of MT in spiked synthetic saliva and urine samples [36,37]. Sample aliquots (1.5 mL) were spiked with MT to obtain a final concentration of 1.0 $x10^{-5}$ mol L^{-1}. From each sample stock solution, dilutions (1:1 and 1:10) were made using 0.1 mol L^{-1} phosphate buffer, pH=5.0, and submitted to square wave measurements. The hormone content in these samples was quantified using the external calibration method.

3. RESULTS AND DISCUSSION

3.1. Voltammetric Behavior of Melatonin using SPE

The voltammetric behavior of 10.0 μmol L^{-1} MT using the proposed SPE was investigated by CV and SWV in a potential range of 0.0 to 1.0 V (*vs.* Ag/AgCl). From Figures 2A-B, it is possible to observe the presence of faradaic processes related to melatonin oxidation, using CV and SWV, respectively. The oxidation mechanism process involves two stages of electronic transfer [8,38]. Simply, the first electron transferring reaches the formation of a cation radical. This is further oxidized by the loss of a second electron and a proton, so the quinone is formed as a final product, as shown in the reactions of Figure 2C. During the potential reverse scan, no reduction peaks were observed in this condition, which is consistent with the monitored process of the MT [9,39]. Also, the effect of the scan rate on the oxidation peak current (I_{PA}) has shown a

linear dependence between peak current and scan rate suggesting an adsorptive-controlled process [9,38].

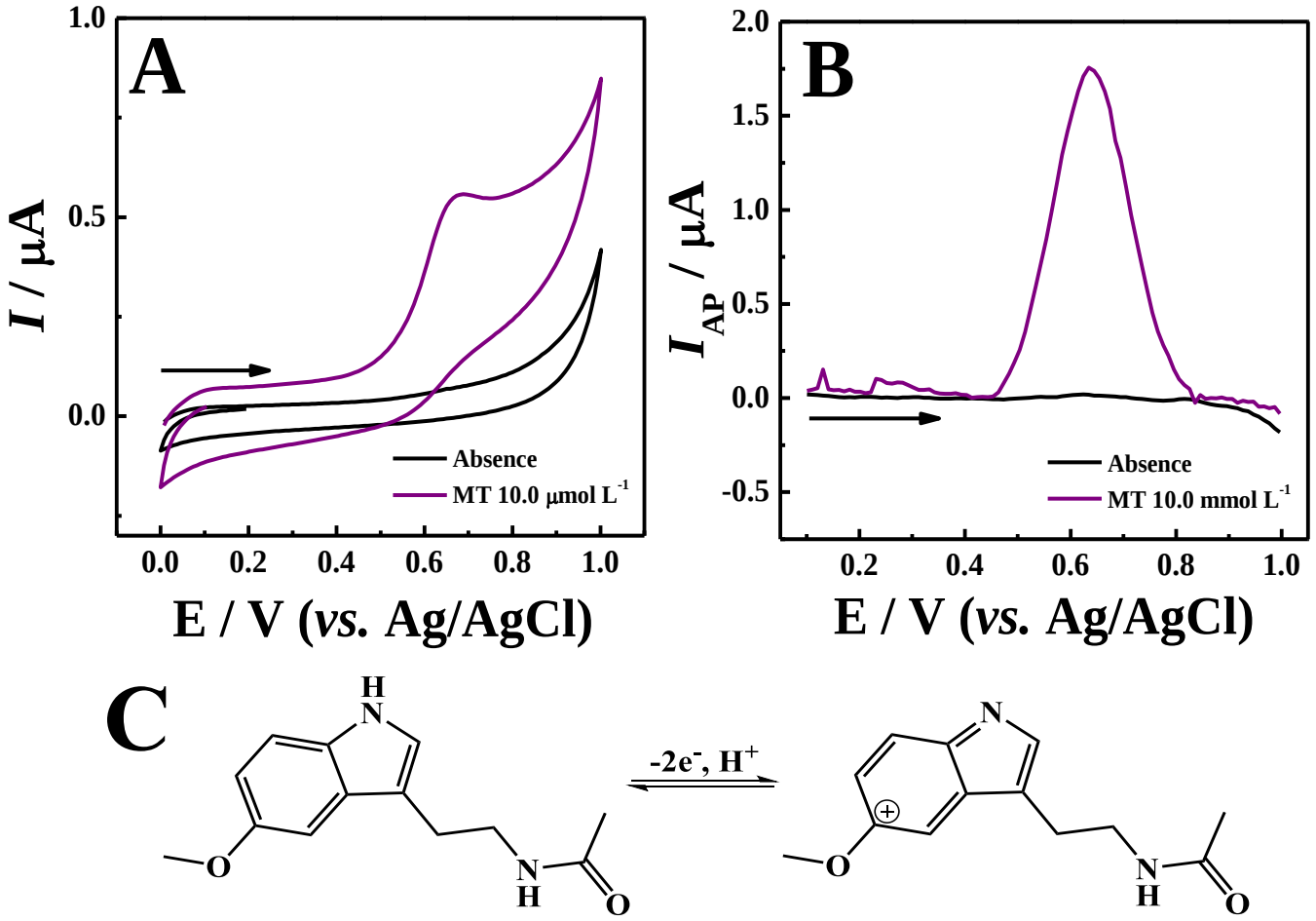

Figure 2. Melatonin voltammetric behavior (Black line in the absence, Purple Line in the presence of MT) using the proposed SPE under A) Cyclic Voltammetric and B) Square Wave Voltammetric conditions. C) Simplified oxidation mechanism for Melatonin oxidation. Experimental conditions: C_{MT} = 10.0 µmol L^{-1}; υ = 50.0 mV.

SWV and EIS measurements were carried out in presence of MT using both commercial and lab-made SPE to compare the electrochemical performance of these devices toward melatonin oxidation (Fig 3). From the SW voltammogram (Fig 3A) recorded using lab-made SPE is possible to observe a shift of potential anodic peak around 50 mV to more positive potential values (E_{AP} = 0.62 V), which could be considered a good result when compared to a commercial electrode (E_{AP} = 0.57 V). These

peak potential values are quite consistent with other works described in the literature, which use lab-made electrodes for the determination of MT. For example, Kumar et al. [12] describe the presence of the melatonin oxidation peak around 0.65 V, as in the work described by Camargo et al. [28], the peak potential value for MT oxidation is around 0.70 V. This displacement of the peak potential does not significantly affect the selectivity of the electrochemical response since the faradaic signals were recorded in a similar potential range. Anodic current peaks registered using the proposed electrode were slightly lower showing a similar sensibility against melatonin detection.

In agreement with the voltammetric experiments, EIS results showed a better electrochemical performance using commercial SPE (Fig 3B). The EIS results were fitted by use of a simple equivalent circuit (Randles-based circuit, insert) which consists of the ohmic resistance (Rs) of the electrolyte solution, the double-layer capacitance (Cdl, which was replaced by a constant phase element), electron-transfer resistance (Rct), and the Warburg element impedance (Zw) was replaced by another CPE (C - as the Warburg N value is fixed at 1/2 so that the circuit is modeled correctly, it is customary to use a CPE, whose coefficient value is variable, allowing adjustment of the circuit for the simulation of the equivalent circuit for systems in which the value of 1/2 is not favorable, that is, systems not ideal and not limited by diffusion [40]. Nyquist diagram obtained in the presence of melatonin provided values of electron-transfer resistance of 764Ω (CPE_1:N=0.83; CPE_2: N=0.43) and 2.28kΩ (CPE_1:N=0.89; CPE_2: N=0.35) for commercial and lab-made screen-printed electrodes, respectively. Considering that the proposed electrode has shown a good electrochemical performance towards melatonin oxidation it was used in the development of an electroanalytical method.

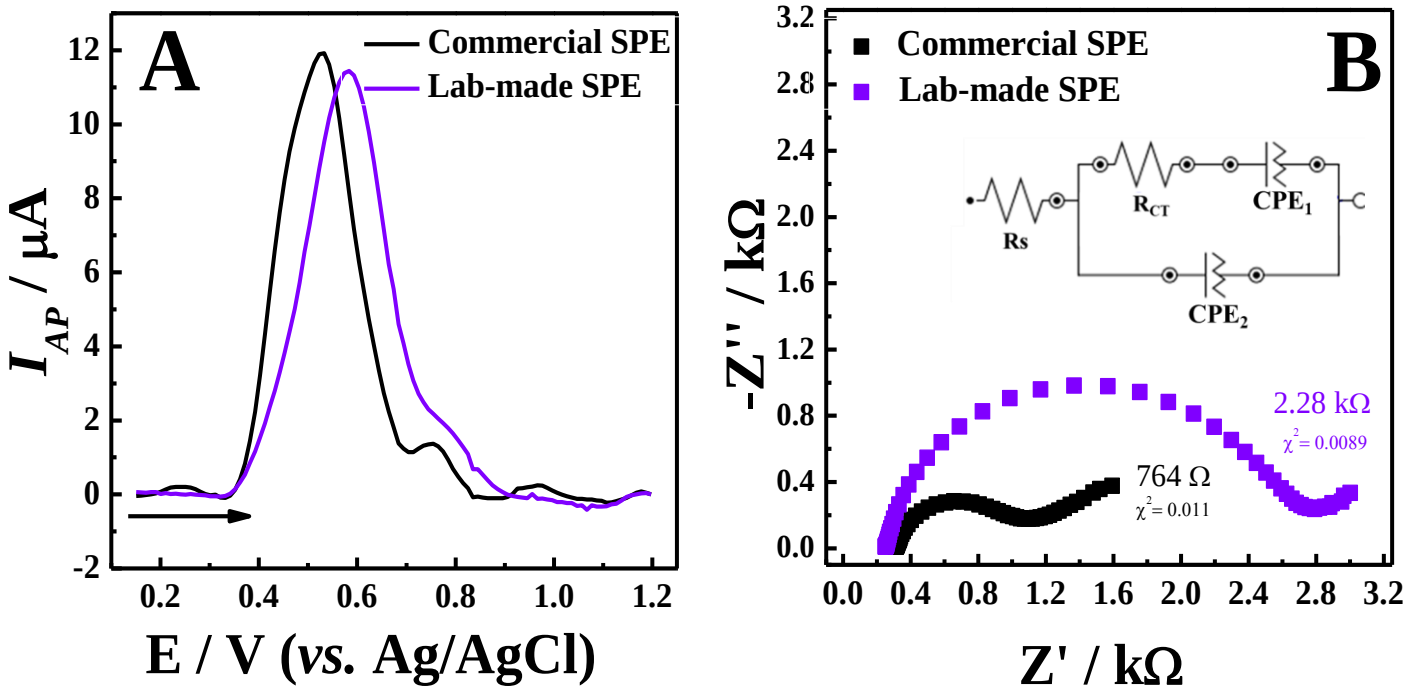

Figure 3. A) Baseline corrected square wave voltammograms obtained for Commercial and Lab made SPE in the presence of 75 µmol L^{-1} MT. B) Nyquist diagram obtained for Commercial, and lab-made SPE in the presence of 75 µmol L^{-1} of MT under potential peak condition, a frequency range of 100 kHz to 1 mHz and amplitude of 10 mV with 10 data points per frequency decade.

3.2. Methodology Optimization and Analytical performance

Initially, a pH variation study was performed to evaluate MT behavior in different conditions. The effect of phosphate buffer pH on square wave voltammograms of MT (Fig.4A) shown that at pH 5.0, higher anodic peak currents were obtained. Fig. 4B (purple curve) shows a variation of anodic peak potentials for MT with pH were fitted with following equation: E_{AP} (V) = -0.033 pH + 0.823. The results showed that the oxidation potential of MT shifts to a less positive potential with increasing pH solution, which is a consequence of the deprotonation involved in the oxidation process that is facilitated at higher pH values. The slope of 0.033 V pH^{-1} suggests a transfer of two electrons to each proton during the oxidation of MT, as predicted in the literature [9].

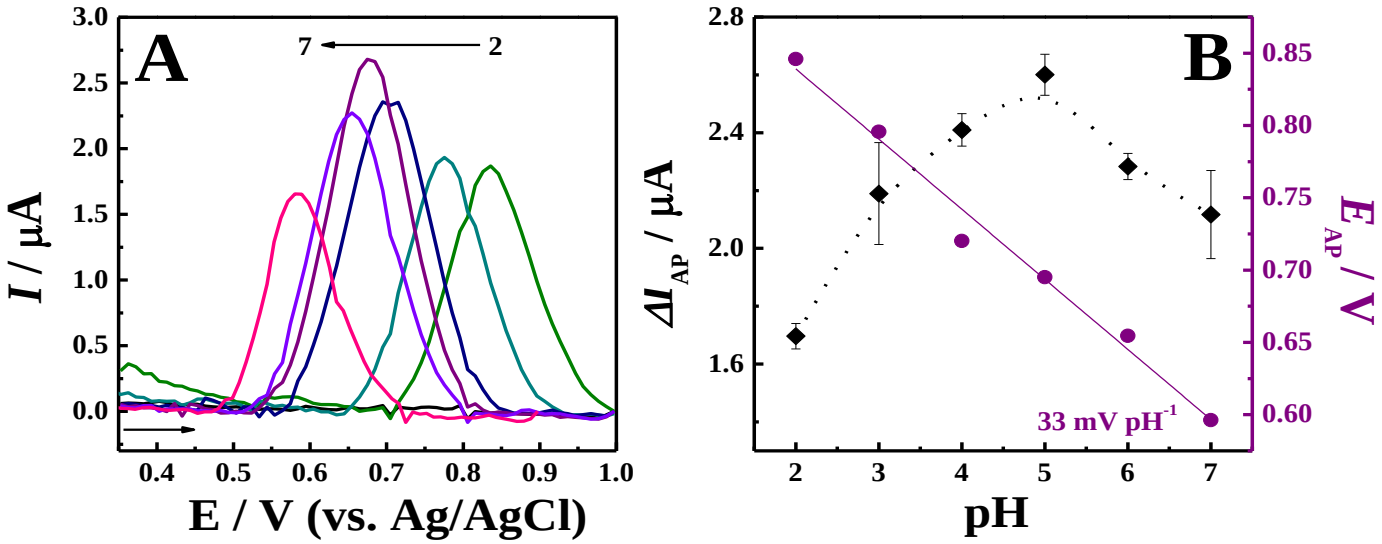

Figure 4. A) Baseline corrected square wave voltammograms obtained for pH study; and B) Anodic current and potential peak versus pH electrolyte solution. C_{MT} = 10.0 µmol L^{-1}; f = 75 Hz; Δ_{EP} = 50 mV; Δ_{ES} = 10 mV; Electrolyte Phosphate Buffer.

The parameters of the voltammetric technique, SWV, were also optimized, reaching the maximum current value with a frequency of 75 Hz, pulse amplitude of 50 mV, and step of 10 mV.

With all the parameters of the proposed methodology already optimized, it was possible to obtain an analytical curve for melatonin using the proposed device. Fig. 5A shows well-defined voltammetric peaks, which yielded a linear increase in the current response as an MT concentration increase in a range of 0.250 to 75.0 µmol L^{-1} (Fig. 5B). The linear regression function for MT was I_{AP} (µA) = 0.168C_{MT} (µmol L^{-1}) + 0.0869, showing a good correlation (R^2 = 0.99), and a good sensitivity. The calculated detection limit (LOD – 3Sd/Slope) and quantification limit (LOQ – 10Sd/Slope), were 25.8 and 83.3 nmol L^{-1}, respectively [41].

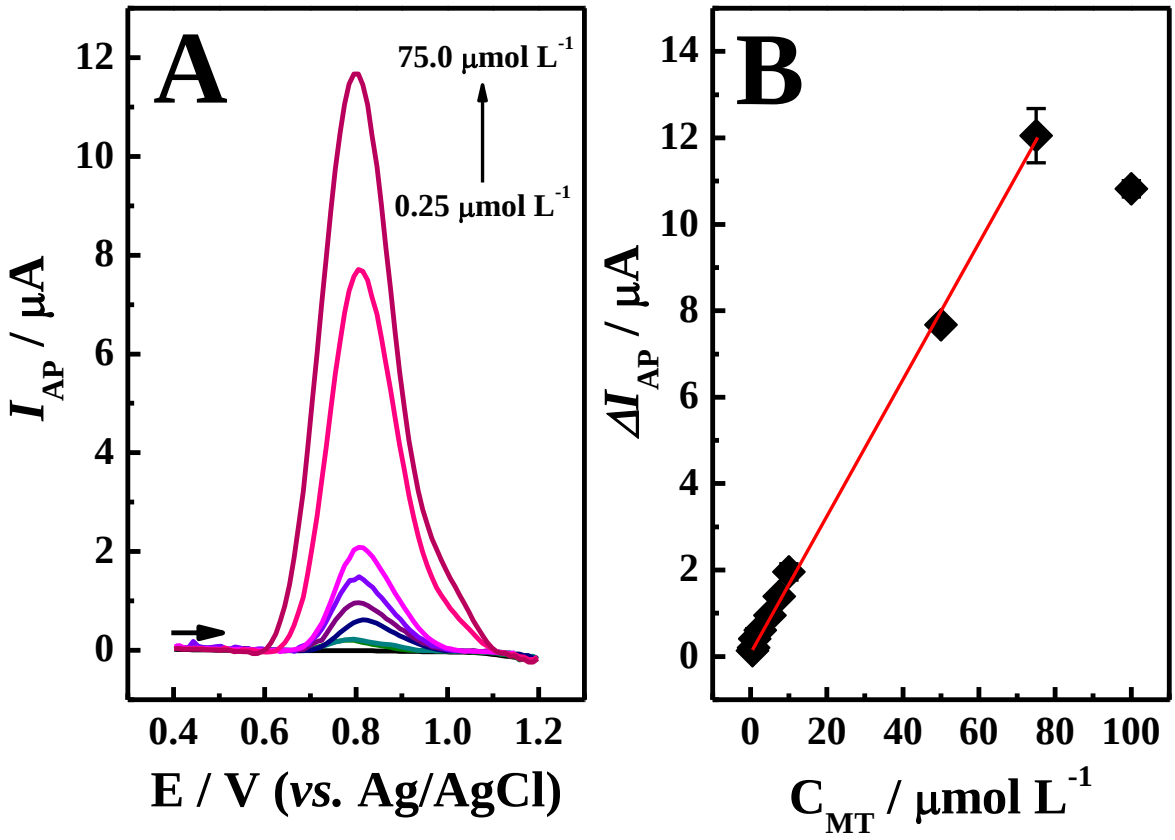

Figure 5. A) Baseline corrected square wave voltammograms obtained using the SPE for successive additions of MT in the range of 0.25 to 75.0 µmol L^{-1}. B) A linear relationship between anodic peak current and MT concentration (n=3). Experimental conditions: f = 75 Hz; Δ_{EP} = 50 mV; Δ_{ES} = 10 mV; Electrolyte 0.1 mol L^{-1} Phosphate Buffer pH = 5.0.

To assess the robustness and agreement of the responses obtained by the proposed sensor (Fig. 6A-B), six consecutive measurements were made using the same electrode surface (repeatability), as well as measurements with six different SPE, from different print batches (reproducibility). Both studies were performed using an MT concentration of 10.0 µmol $L^{-1,}$ and a relative standard deviation of 2.4 and 12.0% were obtained for both studies, respectively, indicating that even varying the electrode surface, the responses obtained are consistent with each other. As was observed (Fig. 6A), a signal

drop, and then on a signal stabilization, for all studies, the current values of the 5th SWV scan were used.

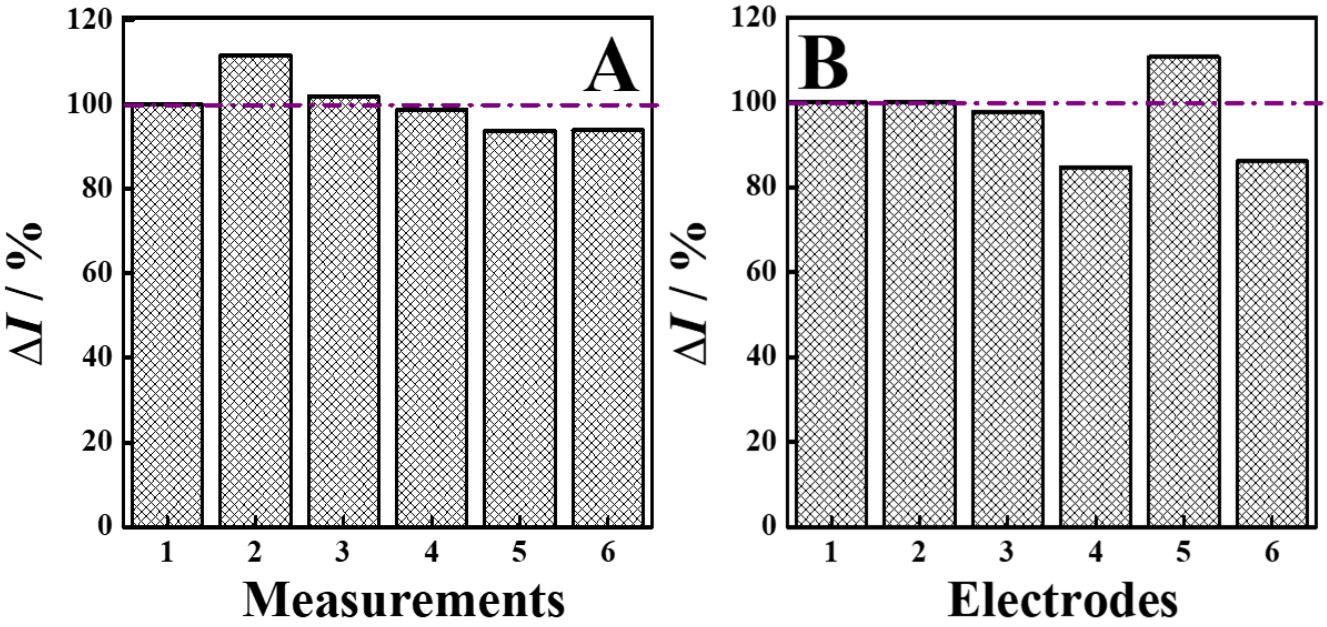

Figure 6. Bar graphs for A) Repeatability (consecutive measurements using the same electrode surface) and B) Reproducibility (measurements using different electrodes) study. C_{MT} = 10.0 µmol L^{-1}; f = 75 Hz; Δ_{EP} = 50 mV; Δ_{ES} = 10 mV; Electrolyte: 0.1 mol L^{-1} Phosphate Buffer pH = 5.0.

Table 1 shows a comparison between other devices described in the literature, for MT electrochemical determination. From the features presented by the devices described in, the proposed SPE showed satisfactory results and often superior to other devices. It is worth mentioning that the initial objective is to show the versatility of application of the SPE, and that even though it is a simple construction device, of low cost, and without modifying species on its surface, neither its application nor its performance is affected by the simplicity of its characteristics. The use of modifiers can indeed improve different aspects of the device; however, sometimes it can become laborious and expensive and is not practical in everyday applications. Therefore, the use of simpler systems can meet demands with similar performance.

Table 1. Comparison of some characteristics of different electrodes for the determination of Melatonin with the proposed electrode

Electrode	Sample	Sensitivity / µA L µmol^{-1}	LDR / µmol L^{-1}	LOD / nmol L^{-1}	REF
SPE	Serum – Saliva – Urine*	0.168	0.25 to 75	25.8	***
GPT/WPE[1]	Saliva – Urine*	0.0583	0.8 to 100.0	32.5	[28]
SnO_2-Co_3O_4@ rGO/CPE[2]	Serum – Tablet	-	0.02 to 6.0	4.1	[8]
FeCo@CNFs/GCE[3]	Serum – Tablet	0.022	0.05 to 250.0	2.7	[42]
MagNPs/Cdots/ GCE[4]	-	0.38	0.05 to 13.5	4.4	[43]
AHNS:PdNPs:ErGO/GCE[5]	Urine - Tablet	0.12	5 to 100.0	90.0	[11]

***This work; GCE - Glassy carbon Electrode; [1]Waterproof paper electrodes; [2]Ionic liquid carbon paste electrode modified with reduced graphene oxides decorated with SnO_2-Co_3O_4 nanoparticles; [3]Carbon nanofibers arrays decorated with FeCo alloy; [4]Nano-magnetic Fe_3O_4 nanoparticles decorated with carbon quantum dots; [5]Polymer nanocomposite between Palladium Nanoparticles, AHSN and Electrochemically reduce graphene oxide; *Synthetic samples.

3.3. Samples Analysis

The simultaneous determination of melatonin in the presence of other biologically relevant compounds which may be present in the samples was studied. Cortisol (CORT), Glucose (GLU), Urea (UR), Estradiol (ET) were used in the same concentration of MT, 5.0 µmol L^{-1}, and no significant signal interference was observed (Fig. 7).

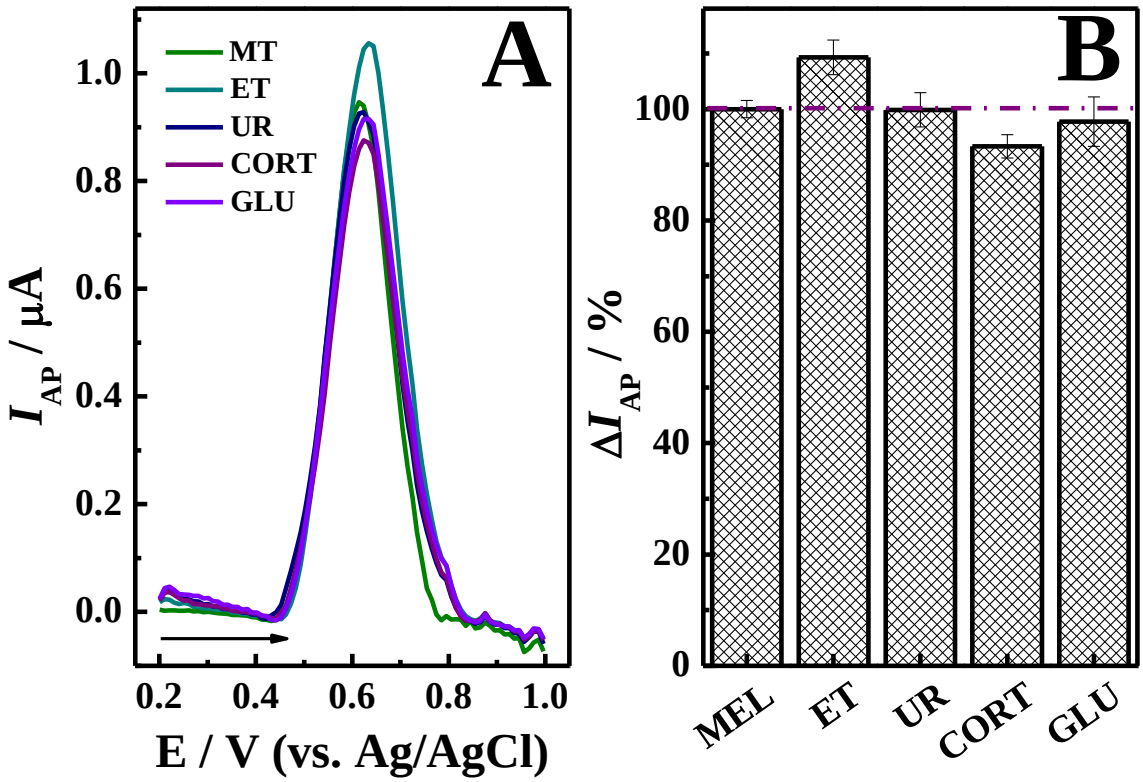

Figure 7. A) Baseline corrected square wave voltammograms; and B) Bar graph for current variation comparison obtained for concomitant study. C_{MT} = 5.0 µmol L^{-1}; f = 75 Hz; Δ_{EP} = 50 mV; Δ_{ES} = 10 mV; Electrolyte: 0.1 mol L^{-1} Phosphate Buffer pH = 5.0. ET: estradiol; GLU: glucose; CORT: cortisol; UR: urea.

To examine the performance of the proposed method, artificial urine, and saliva samples were spiked with MT, then were diluted using Phosphate buffer solution at pH = 5.0, reaching two different MT levels, for both samples. The determination results are reported in Table 2. The good recovery percentage reveals that the proposed SPE has an excellent capability for accurate determination of MT in different real samples.

Table 2. Results obtained for Sample Analysis

Sample	Dilution	Expected / μmol L^{-1}	Found / μmol L^{-1}	Rec / %
Urine	1:1	5.00	5.20±0.04	104.0
	1:10	1.00	1.02±0.03	102.4
Saliva	1:1	5.00	4.80±0.08	95.6
	1:10	1.00	1.01±0.02	101.2

Conclusions

In the current study, a simple lab-made screen-printed electrode (SPE) was built adopting a simple and feasible printing strategy. The proposed electrode arrangement was constructed using low-cost materials, such as graphite powder and colorless nail polish. Excellent electrochemical performance of SPE was observed against melatonin oxidation and it was used for the development of a simple methodology for hormone determination in urine and saliva spiked samples. Under optimized experimental and instrumental conditions, significative figures of merits (LDR, LOD, and LOQ) were achieved. Also, the analytical performance was compared with the earlier literature reports demonstrating that even a simple device can be applied for several determinations, presenting good and comparable results with more elaborate devices.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ava Gevaerd: Data curation, Investigation, Methodology, Writing - original draft. **Emily Y. Watanabe**: Data curation, Investigation, Methodology. **Bruno C. Janegitz**: Conceptualization, Supervision. **Márcio F. Bergamini**: Conceptualization, Funding acquisition, Investigation, Methodology, Supervision. **Luiz H. Marcolino Júnior**: Conceptualization, Funding acquisition, Investigation, Methodology, Supervision.

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