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# Enhancement of amperometric response to tryptophan by proton relay effect of chitosan adsorbed on glassy carbon electrode

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ABSTRACT

A simply prepared, low-cost, and sensitive electrochemically activated glassy carbon electrode ($GCE_a$) modified with absorbed chitosan (CHIT) film for quantification of tryptophan (Trp) is reported. Combination of cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) techniques were used for characterization of the electro-oxidation of the amino acid and the electro-analytical performance of the CHIT-modified electrode. The electro-oxidation of Trp involves an irreversible two-electron and two-proton transfer process in both bare and modified electrodes, but the adsorption of CHIT as a polycation onto $GCE_a$ produces a ≈4-fold increase of the oxidation current of Trp without changing both the oxidation potential and the heterogeneous reaction rate constant, suggesting that the biopolymer behaves as a proton relay species, probably due to hydrogen bonding/proton acceptor capability of hydroxyl and ether groups of CHIT. Finally, the electro-analytical features of the CHIT-modified electrode as Trp sensor were also evaluated, obtaining a linear response range up to 130 μM Trp, sensitivity of 0.68 μA $\mu M^{-1}$ and detection limit of 0.04 μM Trp, with almost no interference of other amino acids.



## 1. Introduction

Tryptophan (Trp) is an essential amino acid, with a significant role in cell metabolism as a protein building block and in the synthesis of neurotransmitters, such as serotonin [1]. Therefore, depletion of Trp in the human body would cause low levels of serotonin and this may be involved in rapid fluctuations in mood, depression, aggression and in blocking the analgesic effect of morphine [2]. Moreover, Trp deficiency has also been associated with Alzheimer Disease (AD), since it was shown that increasing Trp intake would decrease pathological plaque in AD [3]. On the other hand, toxic products generated in the brain by improper metabolism of Trp could cause hallucinations and delusions [2]. Also in animals the absence of Trp produces significant effects, such as delayed growth and maturation of the central nervous system in rats [4], as well as affecting the thyroid gland and energy waste in chickens [5].

Therefore, the development of sensitive, rapid and reliable analytical methods for the quantification of Trp is a current issue for its application in both health and dietary aspects of humans and animals. Several conventional analytical techniques such as high-performance liquid chromatography (HPLC) [6], liquid chromatography-tandem mass spectrometry [7], spectrophotometry [8], spectrofluorometry [9] and capillary electrophoresis [10] have been widely used for the detection and quantification of Trp in different type of samples. However, most of these techniques have the disadvantages of requiring expensive and not always available equipment and in some cases a complex and time-consuming pretreatment of the sample is needed.

On the other hand, electrochemical techniques have been widely used for the detection and quantification of a variety of standard and real samples including amino acids, and are characterized by its simplicity, precision and sensitivity [11,12]. However, the electrochemical response of Trp is not always satisfactory, mainly because of the slow rate of heterogeneous electron transfer at the electrode surface [13]. In order to overcome this disadvantage, several kinds of nano-structured modified electrodes have been used to

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improve the electrochemical response of Trp, such as the incorporation of multi-walled carbon nanotube/cobalt salophen [14], electrospun carbon nanofibers [12], or silver nanoflakes deposited on the surface of molybdenum sulfide [15]. Albeit these modified electrodes exhibit a good performance for electrochemical detection of Trp, most of them require laborious procedures to modify the electrode surface, e.g. synthesis of nanoparticles or nanocomposites.

As a different approach, in the recent years the natural biopolymer Chitosan (CHIT), which is obtained from deacetylated chitins, has begun to be used for the modification of electroactive surfaces [16–18]. This biomolecule is a primary aliphatic amine derivative [19], and under specific conditions it is able to form highly water swellable hydrogels [20]. Recently, Seng et al. [17] have shown that acetylene black paste electrodes (ABPE) modified with either CHIT or salicylaldehyde modified chitosan (s-CHIT) increased the anodic stripping current of Trp compared with both ABPE and carbon paste bare electrodes. However, despite the detection improvement, the role of CHIT on the reaction mechanism of electro-oxidation of Trp remained elusive.

In this work, we describe the electro-analytical response of a low-cost CHIT-modified glassy carbon electrode prepared by a simple and quick procedure. The electro-oxidation of Trp was characterized with both bare and CHIT-modified electrodes as function of pH, and it was confirmed that the single voltammetric oxidation peak is produced by a two electron and two proton irreversible reactions [21], independently of the presence of adsorbed CHIT. However, the presence of the biopolymer produces an almost 4-fold increment of the anodic peak current without modification of both the oxidation potential and the heterogeneous reaction rate constant, suggesting that adsorbed CHIT acts as a proton relay species rather than as a catalyst. Additionally, the electro-analytical parameters for detection of Trp with the CHIT-modified electrode were characterized in detail, including the interference effect of several amino acids, and its analytical performance for quantification of Trp in a commercial pharmaceutical formula.

## 2. Experimental

### 2.1. Materials

Chitosan (CHIT) from crab shells (minimum 85% deacetylated), tryptophan (Trp) and potassium ferricyanide $K_3[Fe(CN)_6]$ (99%) were purchased from Sigma–Aldrich (Argentina S.A.). Potassium ferrocyanide $K_4[Fe(CN)_6]$ and acetic acid were from Cicarelli (Argentina). Phosphate buffer solution ($2 < pH < 12$) was made by mixing reagent grade phosphoric acid ($H_3PO_4$), dibasic ($Na_2HPO_4$) and/or monobasic ($NaH_2PO_4$) sodium phosphate salts from J.T. Baker (Mexico D.F., Mexico). All other reagents (analytical grade) were from Parafarm (Argentina) and used without further purification. Dietary supplement capsules of 500 mg of L-tryptophan (Vitabay®, VB 1057) were purchased in a local pharmacy. Triply distilled water was used for all solutions, which were deoxygenated by bubbling for at least 15 min with high-purity nitrogen (99.99% Indura, Argentina) prior to electrochemical measurements and keeping the gas flow over the solution during the experiments.

### 2.2. Methods

#### 2.2.1. UV–vis absorption and fluorescence measurements

UV–vis absorption spectra were registered using an Agilent 8453 diode array spectrophotometer (Palo Alto, CA, USA). Fluorescence emission and anisotropy measurements were achieved with a Hitachi F-2500 spectrofluorometer (Kyoto, Japan). Excitation of Trp solutions was performed at 280 nm and both emission spectrum and anisotropy was measured in the 300–500 nm spectral range. All measurements were performed with continuous stirring using a magnetic bar and at constant temperature of 25 °C. Anisotropy measurements were performed using the classical L-format and calculated as described before [22].

#### 2.2.2. Electrochemical measurements

Cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) studies were carried out with an Autolab PGSTAT 30 potentiostat using the software package GPES and FRA 4.9 (Eco-Chemie, Utrecht, Netherlands) [23]. Electrochemical experiments were performed in a three-compartment electrochemical cell with standard taper joints thus all compartments could be hermetically sealed with Teflon® adapters. Working electrodes were prepared with glassy carbon disks, diameter 2 mm (Metrohm, 6.1204.600). A large-area platinum wire was used as a counter electrode. The potentials were measured against a reference electrode $Ag|AgCl|Cl^-$ (3 M) and all measurements were performed at room temperature (25° C). For the EIS experiments, the sine wave potential amplitude applied was 5 mV at a bias potential of 200 mV and frequency range 0.05 Hz–10 KHz. Experimental EIS curves were fitted by the non-linear least squares method provided by Autolab FRA 4.9® software comparing the experimental system to a Randles equivalent circuit (Fig. S1 of Supplementary Information). DPV measurements were performed from 0.60 to 1.2 V at 0.020 V $s^{-1}$ with potential pulse amplitude of 0.050 V and data sampling width of 0.010 V.

#### 2.2.3. Preparation of the CHIT-modified electrode

The surface of the glassy carbon electrodes (GCE) was polished sequentially with alumina powder (Buehler, USA) of decreasing particle size of 1.0, 0.3 and 0.05 μm, and thoroughly rinsed with triply distilled water and sonicated for 1 min between polishing stages. Prior to the adsorption of the chitosan layer, electrochemical activation of the polished GCE was performed as follows: the electrode was cleaned in 0.1 M sodium phosphate buffer solution at pH 7 by cyclic voltammetry between −0.50 and +1.2 V at 0.050 V $s^{-1}$ until a stable voltammogram profile was observed. Subsequently, an oxidation potential of 2.0 V for 60 s followed by a pulse reduction at −1.1 V for 30 s also in a solution of 0.1 M phosphate buffer at pH 7 was applied. Afterwards, the electrode surface was rinsed with triply distilled water and dried under nitrogen flow. The electrochemical activated electrode was immediately used for its modification with the chitosan film.

The adsorption of CHIT film on the previously activated glassy carbon electrode ($GCE_a$) was formed by dipping the activated electrode in a 0.5 wt.% CHIT dissolved in 2 wt.% acetic acid solution. The optimal time for chitosan film adsorption was ≥20 min, as tested by the constant value of the oxidation peak current of a 100 μM Trp standard solution in 0.1 M phosphate buffer pH 6.0 (±0.1) obtained by cyclic voltammetry at 0.050 V $s^{-1}$. The CHIT-modified electrode ($CHI/GCE_a$) also was immediately used after preparation.

#### 2.2.4. Real sample preparation for Trp quantification

Commercial tablets formulation containing 500 mg Trp (Vitabay® VB 1057) were purchased in a local pharmacy. Tablets were triturated and mixed thoroughly during 1 h with 10 ml of 0.1 M phosphate buffer (pH 2.0). Then, the suspension was filtered, and the collected solution was diluted to 100 ml with the same buffer. Subsequently, a 15 μl aliquot of the diluted solution was added to the electrochemical cell containing 10 ml of the phosphate buffer.

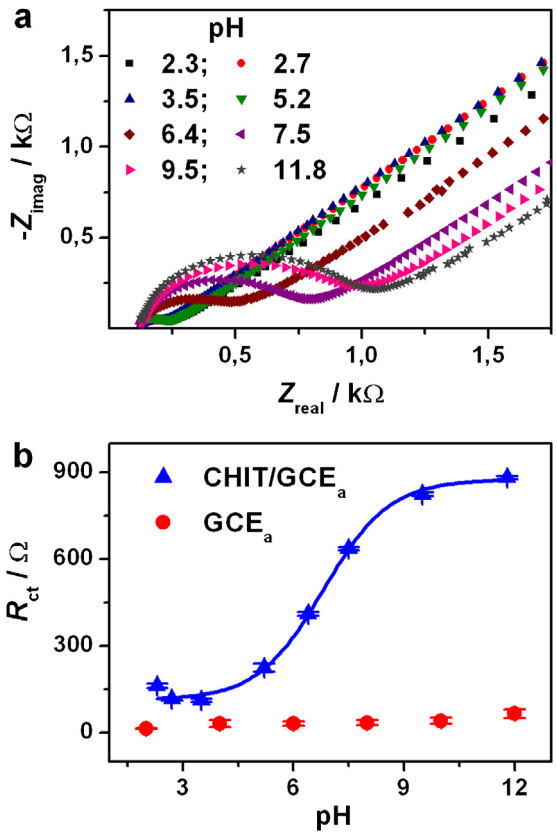



**Fig. 1.** (a) Nyquist plots of electrochemical activated GCE modified with CHIT (CHIT/$GCE_a$) in presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M phosphate buffer solution at several pH values. The sine wave potential amplitude applied was 5 mV, at a bias potential of 200 mV and frequency range 0.05 Hz–10 KHz. (b) Charge transfer resistance $R_{ct}$ values as a function of pH for: bare $GCE_a$ (●) and CHIT/$GCE_a$ (▲).

## 3. Results and discussion

### 3.1. Electrochemical characterization of CHIT-modified glassy carbon electrode

Electrochemical impedance spectroscopy (EIS) experiments as a function of pH using equimolar (5 mM) solution of the redox couple $[Fe(CN)_6]^{3-/4-}$ in 0.1 M phosphate buffer were performed to explore the interfacial electrical properties of the electrochemical activated bare glassy carbon electrode ($GCE_a$) and CHIT-modified one (CHIT/$GCE_a$). This technique is suitable for characterization of intrinsic features of the material or specific processes which may affect the conductivity of an electrochemical system [23,24]. Fig. 1a shows the pH dependence of the impedance spectra obtained with CHIT/$GCE_a$. On the contrary, the impedance spectra obtained with the bare $GCE_a$ were almost not modified with pH (Fig. S2 of Supplementary Information). Nevertheless, in both cases satisfactory fittings of the impedance curves were obtained by modeling with the impedance of the classical Randles circuit, supported in the Autolab FRA 4.9® software, as mentioned in Section 2. This model is the simplest equivalent circuit describing a single-step Faradaic process in the presence of semi-infinite linear diffusion [24]. This circuit model comprises three basic elements: the electrolyte resistance between working and reference electrode ($R_s$), the Warburg impedance ($W$) that reflects the influence of the mass transport of electroactive compounds on the total impedance of the electrochemical cell, and the double-layer capacitance ($Q_{dl}$) represented by a constant phase element because of the non-ideal behavior of the double layer and the Faradaic impedance due to the whole charge transfer process. The latter element includes the charge-transfer resistance ($R_{ct}$) of the electrode [24]. All recovered values for the three circuit elements of both electrodes are presented in Table S1 of Supporting Information. Except for the $R_{ct}$ value obtained with CHIT/$GCE_a$ that show a sigmoidal increases with pH, Fig. 1b, the values of the rest of circuit elements for both electrodes remained almost constant with pH.

The $R_{ct}$ variation with pH observed with the CHIT-modified electrode was fitted with the classical Henderson–Hasselbach equation (solid line in Fig. 1b) yielding a $pK_a = 6.8$, in good agreement with the value reported in homogeneous solution, e.g. $pK_a = 6.3$ [25]. Therefore, the adsorption of CHIT on the electrode surface does not strongly modify the ionization equilibrium of the amino groups, and at pH < 6.8 it can be considered that the biopolymer is adsorbed as a polycation. Moreover, at the same pH range, the ferrocyanide ion is expected to be a negatively charged species, since it behaves as a tetravalent Brønsted base with $pK_1 = -2.5$, $pK_2 = -1.1$, $pK_3 = 2.65$, and $pK_4 = 4.2$ [26]. Consequently, the observed decreases of $R_{ct}$ with decreasing pH can be attributed to electrostatic attractive interactions between the adsorbed polycation film and the negatively charged redox probe. Instead, at pH > 6.8, the adsorbed CHIT becomes deprotonated and the electrostatic attractions are destroyed, resulting in the abrupt increment of the $R_{ct}$ value. On the contrary, for the bare $GCE_a$, preferential protonation at the electrode surface is not possible and therefore the conductivity properties of the electrode remained almost independent on pH. Cruz et al. [16] have reported that the voltammetric peak current for $[Fe(CN)_6]^{4-}$ is enhanced by the presence of CHIT films on the GCE surface, and the effect was explained in terms of a positive partition of the probe driven by hydrogen bonding interactions between the redox probe and CHIT. However, as it is shown in Fig. 1b, attractive electrostatic interactions between positively charged CHIT and the negative redox probe can be also responsible for the current enhancement. Therefore, EIS experiments demonstrate that under acidic conditions CHIT is absorbed as a polycation onto the glassy carbon surface.

The voltammetric peak current ($i_{pa}$) of 250 μM $[Fe(CN)_6]^{4-}$ in 0.1 M phosphate buffer at pH 4.0 obtained with $GCE_a$ and CHIT/$GCE_a$ increased linearly with the square root of sweep rate ($\nu^{1/2}$), suggesting that the electron-transfer reaction at both electrodes is a diffusion-controlled process (Fig. S3 of Supplementary Information). The electrochemical active surface area $A$ ($cm^2$) for each electrode can be estimated, according with Randles–Sevcik Eq. (1):

$$i_{pa} = 2.69 \times 10^5 n^{3/2} D^{1/2} A C \nu^{1/2} \quad (1)$$

where $n$ is the number of exchanged electrons, $D$ the diffusion coefficient ($cm^2\ s^{-1}$), and $C$ is the concentration of electroactive species ($mol\ cm^{-3}$). Thus, from the slope values of the plots of $i_{pa}$ vs. $\nu^{1/2}$ and using $D = 5.9 \times 10^{-5}\ cm^2\ s^{-1}$ [12], the effective electroactive areas of $A = 0.048$ and $0.078\ cm^2$ for $GCE_a$ and CHIT/$GCE_a$ were obtained.

### 3.2. Effect of adsorbed CHIT on the electro-oxidation of Trp

Fig. 2 compares the cyclic voltammograms of 100 μM Trp in 0.1 M phosphate buffer solution at pH 4.0 with a sweep rate of $0.050\ V\ s^{-1}$ obtained with bare non- and electrochemical activated electrodes, i.e. GCE and $GCE_a$, and chitosan modified activated electrode CHIT/$GCE_a$, respectively. The lack of a cathodic peak indicates that the electro-oxidation process of Trp at all electrodes is irreversible, as previously reported for Trp using a pyrolytic graphite electrode (PGE) [21]. The analysis of the anodic peak indicates that the bare GCE presents the highest peak potential ($E_{pa} = 0.901$ V) and lower peak current ($i_{pa} \approx 2$ μA). After electrochemical activation, the oxidation potential of bare and CHIT-modified electrodes was shifted to a lower value, e.g. $E_{pa} = 0.795$ V, indicating that the adsorption of CHIT does not alter the free energy of the electro-oxidation reaction of Trp.

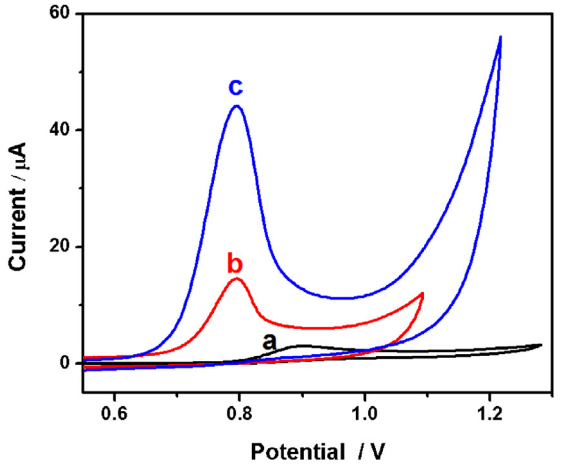



**Fig. 2.** Cyclic voltammograms obtained at scan rate of 50 mV $s^{-1}$ of 100 μM Trp (in 0.1 M phosphate buffer pH 4.0) with glassy carbon electrode (GCE): (a) bare GCE, (b) electro-activated bare GCE ($GCE_a$), and (c) Chitosan modified electrode ($CHI/GCE_a$).

This potential reduction induced by the electrochemical activation treatment is associated with the development of active regions on the glassy carbon surface, such as edge plane defects and oxide formation, producing desirable effects such as higher heterogeneous electron-transfer rate and increased adsorption of organic molecules, respectively [27].

As it is shown in Fig. 2, the $i_{pa}$ was improved more than 3-fold (≈340%) with the $CHIT/GCE_a$ than the bare $GCE_a$. Similar behavior has also been reported for chitosan-modified acetylene black paste electrode (ABPE) [17]. However, the anodic peak current increases was only ≈35% and ≈114% after modification with CHIT and salicylaldehyde derivative CHIT (s-CHIT), respectively, and the effect was attributed to increased hydrogen bonding and π–π interactions between CHIT and Trp [17].

In order to prove the existence of specific interactions between Trp and CHIT, both fluorescence emission anisotropy and spectrum of 45 μM Trp solution in 0.1 M phosphate buffer at pH 4 were compared in the absence and presence of 0.1 wt.% CHIT (Fig. S4 of Supplementary Information). Neither the anisotropy value ($r = 0.071 \pm 0.006$) nor the emission spectra of Trp were modified by the presence of CHIT, ruling out the prevalence of both electrostatic and weak specific interactions between Trp and CHIT. Moreover, at pH 4, Trp is a zwitterion species ($pK_a$'s of 2.46 for RCOOH and 9.41 for $RNH_3^+$) and net electrostatic interaction with the positive polycation should not be expected. Therefore, the role of adsorbed CHIT in the electrochemical oxidation of Trp cannot be associated with specific interactions between the amino acid and the biopolymer.

Fig. 3a shows the large pH effect on the cyclic voltammograms of 25 μM Trp in 0.1 M phosphate buffer recorded at 0.050 V $s^{-1}$ with CHIT-modified electrode. Remarkably, the bare $GCE_a$ also showed the same anodic potential shift to lower values with pH but poor changes of $i_{pa}$ compared with $CHIT/GCE_a$, Fig. 3b. The increment of both the anodic potential and peak current for the electro-oxidation of Trp under more acidic conditions has been previously reported for different types of modified electrodes [13,14,28,29], suggesting the relevant role of protons in the electro-oxidation mechanism of Trp [21]. Furthermore, the $E_{pa}$ decreased linearly with pH according with the Nernst Eq. (2) at 25 °C, corresponding to the redox process $b\mathrm{Red} \rightarrow a\mathrm{Ox} + ne^- + m\mathrm{H}^+$, where $n$ and $m$ are the number of moles of electrons and protons transferred, respectively, while $a$ and $b$ represent the stoichiometric coefficients of the oxidized Ox and reduced Red forms of the analyte, respectively.

$$E_{pa} = E^0 + (0.059/n)\log[(\mathrm{Ox})^a/(\mathrm{Red})^b] - (0.059m/n)\mathrm{pH} \quad (2)$$

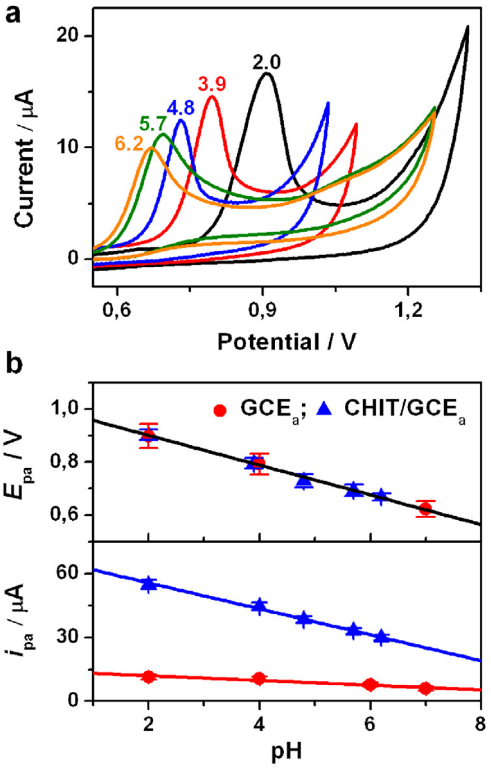



**Fig. 3.** (a) Cyclic voltammograms of 25 μM Trp at $CHIT/GCE_a$ in 0.1 M phosphate buffer solution of different pH, as indicated in the figure. Scan rate: 0.050 V $s^{-1}$. (b) pH dependence of oxidation peak potentials and current $E_{pa}$ and $i_{pa}$, respectively, for the electrochemical activated $GCE_a$ (●) and chitosan modified electrode $CHIT/GCE_a$ (▲).

The linear regression of the plotted potential data of Fig. 3b with Eq. (2) yielded a slope value of −57(±2) mV for both bare and CHIT-modified electrodes, which is practically the same as the expected value of −59 mV at 25 °C if $m/n = 1$. Therefore, within the experimental error, it can be assumed that equal mole number of protons and electrons are transferred in the electrochemical oxidation of Trp at the activated glassy carbon electrode, independently of the presence of the absorbed CHIT film.

In order to estimate the net charge transferred in the reaction, the effect of the sweep rate ($\nu$) on the voltammograms of 100 μM Trp in phosphate buffer at pH 2.0 were analyzed for both bare and modified electrodes. Fig. 4 shows the voltammogram changes elicited using $CHIT/GCE_a$, and in the inset figure is represented the variation of $E_{pa}$ with $\log \nu$ for both electrodes according with Eq. (3) [30]:

$$E_{pa} = E^0 - \frac{2.303RT}{(1-\alpha)nF}\log\frac{RTk_s}{(1-\alpha)nF} + \frac{2.303RT}{(1-\alpha)nF}\log\nu \quad (3)$$

where $E^0$ is the formal standard potential, $\alpha$ the charge-transfer coefficient, $n$ the number of mole of electrons involved in the electrochemical reaction, $F$ the Faraday constant, and $k_s$ is the standard heterogeneous reaction rate constant ($s^{-1}$) [31]. The same linear relationship was obtained for both electrodes indicating that the CHIT film on the electrode surface does not alter either the amount of electrons transferred or the reaction rate constant $k_s$ for the electro-oxidation of Trp. The linear fitting of the data gives a slope of 74(±5) mV ($R^2 = 0.982$), from which a value of $n \approx 2$ was estimated assuming a typical value of $\alpha \approx 0.6$ [15], and from the intercept the value of $k_s = 7 \times 10^3$ $s^{-1}$ was obtained using $E^0 = 1.15$ V (vs. NHE at pH 2) [32]. As far as we know, there are no reported $k_s$ values for the electro-oxidation of adsorbed Trp molecules. However, values of $k_s = 1.5 \times 10^3$ and $3.5 \times 10^2$ $s^{-1}$ have been found for adsorbed

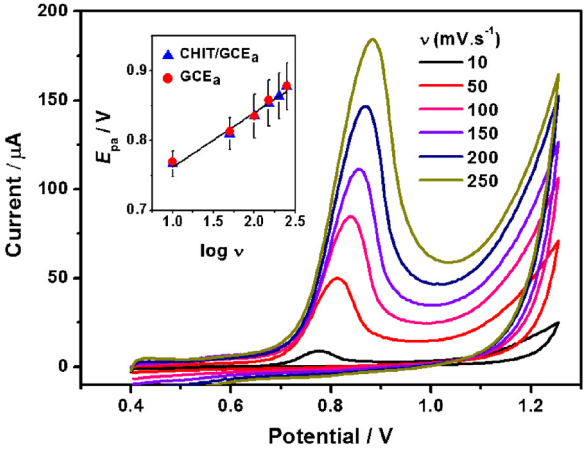



**Fig. 4.** Cyclic voltammograms of 50 μM Trp in 0.1 M phosphate buffer pH 2.0 (±0.1) solutions obtained with the CHIT/$GCE_a$ at several scan rates $\nu$ (mV $s^{-1}$). *Inset*: Peak potentials vs. logarithm of the sweep rate for both bare $GCE_a$ and CHIT/$GCE_a$, Eq. (3).

monolayers of anthraquinone-2,7-disulphonic acid at pH 1.4 and 4.2, respectively [33].

As the ratio $m/n \approx 1$ (Eq. (2)) and $n \approx 2$ (Eq. (3)), it can be confirmed that two mole of protons are also transferred during the electro-oxidation of Trp at the $GCE_a$ and CHIT/$GCE_a$ electrode, as it has been previously postulated for the electro-oxidation of the amino acid at pyrolytic graphite electrodes (PGE) [21]. In this report, a complete mechanism study of the electro-oxidation of Trp was presented, and it was shown that after 2-$e^-$ irreversible oxidation of Trp coupled with the loss of 2-$H^+$, an extremely electrophilic reactive methylene-imine intermediate is formed, which in turn, is attacked by water molecules leading to the formation of indolic derivatives such as oxi- and dioxindolylalanine, traces of kynurenine and oligomeric compounds [21], as summarized in Scheme 1.

Furthermore, in the electro-oxidation of Trp on PGE, the adsorption of both Trp and its oxidation products was produced [21]. In the present case, for both bare and CHIT-modified electrodes the $i_{pa}$ increased linearly with the sweep rate $\nu$ (Fig. S5 of Supplementary Information), suggesting that the electrochemical oxidation of the amino acid is an adsorption controlled process of either Trp or its oxidation products [34]. Furthermore, of peak current function, e.g. $i_{pa} \times \nu^{-1/2}$, also increased with $\nu$ (Fig. S6 of Supplementary Information), confirming the adsorptive behavior of Trp on both $GCE_a$ and CHIT/$GCE_a$ [34].

Although the characterization of the electro-oxidation products of Trp is out of the scope of this research, extra evidence of their formation and adsorption on the surface of both $GCE_a$ and CHIT/$GCE_a$ is given by EIS experiments of Fig. 5, where the Nyquist impedance spectra before and after one-cycle of electrochemical oxidation of Trp are compared. The impedance spectra were fitted using the Randles model circuit, as mentioned before, and the recovered circuit elements for both $GCE_a$ and CHIT/$GCE_a$ are presented in Table S2 of Supplementary Information. The main effect on the conductivity properties of both electrodes is the increases of the $R_{ct}$ value after electro-oxidation of Trp, probably by adsorption of the electrochemical products, blocking the electrode surface and increasing the double layer capacitance. A quantitatively analysis of the $R_{ct}$ values in Table S2 also indicates that the relative resistance increases after Trp oxidation is almost twice for $CGE_a$ than for the modified CHIT/$CGE_a$ electrode. If it is consider that the rate constant of electron-transfer of the redox probe does not change after amino acid oxidation, it can be assumed that $R_{ct}{}^{before}/R_{ct}{}^{after} = A^{after}/A^{before}$ [35]. Thus, the reduction of the electroactive area ($A$) was 67% and 39% for the for $CGE_a$ and CHIT/$CGE_a$ electrode,

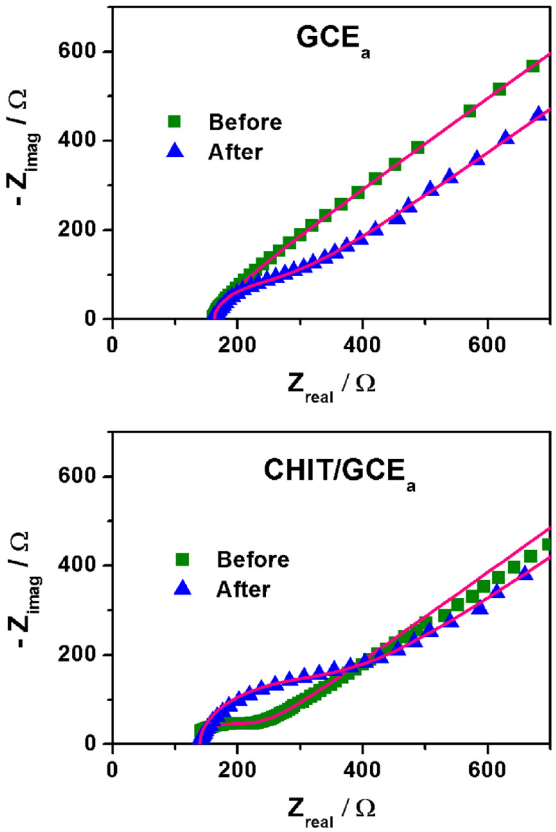



**Fig. 5.** Electrochemical impedance spectrum (EIS) of $GCE_a$ (top) and CHIT/$GCE_a$ (bottom) in presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M phosphate buffer pH 4.0, before (■), and after (▲) Trp oxidation. The sine wave potential amplitude applied was 5 mV, at a bias potential of 200 mV and frequency range 0.05 Hz–10 KHz. Filled lines represents the fitting of the impedance spectra using the Randles circuit model.

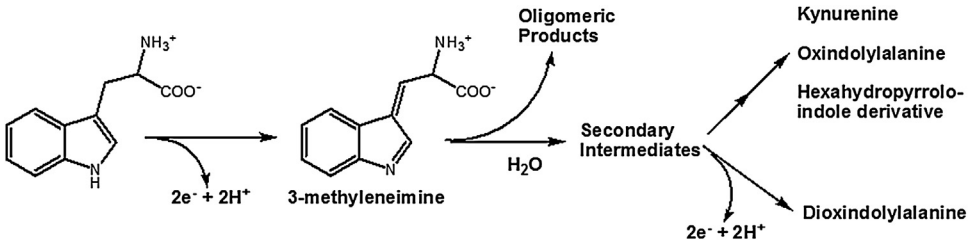
respectively, indicating that the CHIT-modified electrode can be reused 2–3 times before its complete deactivation.

All together, the above results provide evidence that electro-oxidation of Trp at the electro-activated surface of a glassy carbon electrode is an irreversible adsorptive process involving the transfer of 2-$e^-$ and 2-$H^+$, as previously observed [15,21,36,37], and the solely role of the adsorbed CHIT film is to produce a remarkable increase of the anodic peak current. This effect can be expected for electro-oxidation reactions involving coupled proton-transfer and other coupled chemical reactions, which can be catalyzed by a proton acceptor species on the electrode surface [38]. In addition, it has been recently proposed that CHIT produces voltammetric and chronopotentiometric reduction peaks at mercury and solid amalgam electrodes [39]. These peaks were increased by decreasing pH, and were assigned to the catalytic hydrogen evolution reaction, feature associated to compounds with proton donor capacity.

However, results presented suggest that chitosan adsorbed onto glassy carbon surface does not produce a catalytic effect, since the $k_s$ was the same for both bare and CHIT-modified electrodes. Therefore, the anodic current enhancement induced for the CHIT-film for the electro-oxidation of Trp can be associated with the ability of the biopolymer to act as proton relay species, probably by the hydrogen bonding/proton acceptor capability of hydroxyl and ether groups [19,40], since most of the amino groups are protonated at pH < 6.8.

### 3.3. Analytical performance of the CHIT/$GCE_a$

Fig. 6 shows the voltammograms obtained by differential pulse voltammetry (DPV) over a concentration range of 5–130 μM standard Trp solutions in 0.1 M phosphate buffer at pH 2.0. The




**Scheme 1.** Electro-oxidation reaction scheme of Trp adapted from [21].

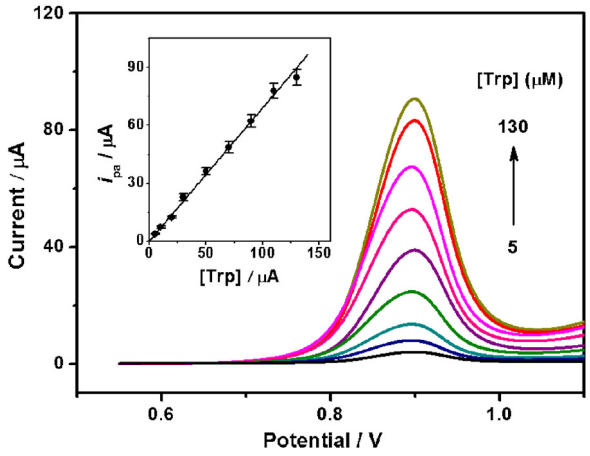



**Fig. 6.** Differential pulse voltammograms as a function of Trp concentration between 5 and 130 μM solutions in 0.1 M phosphate buffer pH 2.0(±0.1). *Inset*: corresponding calibration plot of peak current vs. Trp concentration.

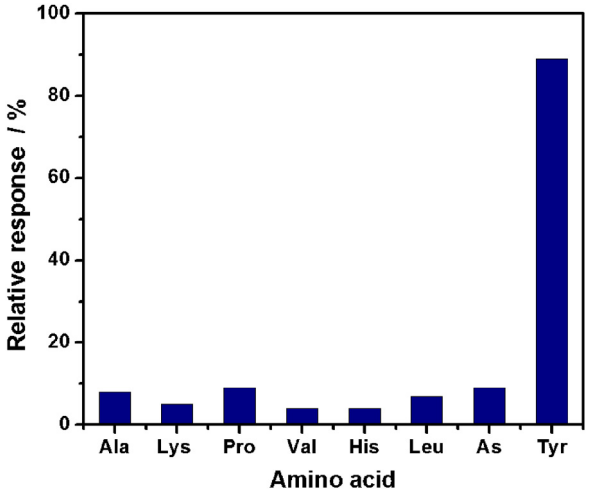



**Fig. 7.** Relative electro-analytical response of the CHIT/GCE$_a$ to other amino acids at concentration 50-fold larger than 25 μM of Trp in 0.1 M phosphate buffer pH 2.0(±0.1). The relative response was calculated as $[1-(i_{pa}{}^{AA+Trp}/i_{pa}{}^{Trp})] \times 100$.

respective analytical calibration curve is represented in the inset of Fig. 6, which is completely linear ($R^2 = 0.998$) in the concentration range studied, with an slope of 0.68(±0.01) μA μM$^{-1}$. Additionally, a detection limit of 0.04 μM Trp was calculated using the criterion of 3× S.D./*s* [41], where S.D. is the standard deviation of the average current obtained after at least five measurements ($n = 5$) of 20 μM Trp standard solutions, and *s* is the sensitivity given by the experimental slope value of the current-concentration calibration curve. The electrode reproducibility of ±5% was calculated from the standard deviation of three different prepared electrodes by measuring the DPV current response of 25 μM Trp standard solution in 0.1 M phosphate buffer at pH 2.0. Furthermore, a set of CHIT/CGE$_a$ electrodes prepared under identical conditions and stored under air at fridge temperature during a week showed similar $i_{pa}$ values (±3%) for the electro-oxidation response of 30 μM Trp in buffer solution at pH 2. This result indicates that the adsorbed CHIT-film remains stable under cold storage conditions and it can be used without additional treatment.

In order to compare the analytical performance of the CHIT/GCE$_a$ with some modified electrodes reported elsewhere [13,29,42–45], the most significant analytical parameters are collected in Table 1.

The linear range, detection limit, and sensitivity of Trp detection with CHIT/GCE$_a$ indicates that this easily prepared electrode presents a very good analytical performance, making it suitable for applications in real samples.

The selectivity of the CHIT/GCE$_a$ to Trp relative to other amino acids commonly present together in real samples, such as alanine, valine, leucine, lysine, histidine, asparagine, proline and tyrosine was also evaluated recording the DPV profiles of 25 μM Trp solutions in presence of each interference compounds at 50-fold larger concentration. Fig. 7 shows the relative percentage response (%*R*) of the electrode considering the Trp solution without interference as control, e.g. $\%R = [1 - (i_{pa}{}^{AA+Trp}/i_{pa}{}^{Trp})] \times 100$ [29]. Except for tyrosine, the rest of the analyzed interferences at 50-fold larger concentration than Trp showed no significant interference (≤10%) on the current response for the amino acid. Nevertheless, for equimolar Tyr-Trp solutions, the interference by Tyr can be considered ≤2%. These results also suggest a remarkable selectivity of the CHIT/GCE towards the electrochemical oxidation of the Trp in presence of other amino acids.

**Table 1**
Comparison of the electro-analytical properties of tryptophan (Trp) detection by different carbon based modified electrodes.

| Modified electrode | Linear range (μM) | Detection limit (μM) | Sensitivity (μA μM$^{-1}$) | Reference |
|---|---|---|---|---|
| Nafion/$TiO_2$-graphene/GCE | 5–140 | 0.7 | 0.08 | [34] |
| Ag@C core–shell nanocomposite/GCE | 0.1–100 | 0.04 | 0.05 | [40] |
| Pencil graphite electrode | 0.5–50 | 0.05 | 1.40 | [41] |
| AuNP- in presence of sodium dodecylbenzene sulfonate/GCE | 0.09–50.0 | | | [29] |
| 4-aminobenzoic acid polymer film/GCE | 1–100 | 0.2 | 0.28 | [13] |
| MWNTs bridged mesocellular graphene | 5–30 | 0.87 | 0.26 | [42] |
| | 60 - 500 | | 0.04 | |
| Chitosan film/GCE$_a$ | 0.1–130 | 0.04 | 0.68 | This work |

GCE, glassy carbon electrode; GCE$_a$, glassy carbon electrode activated, AuNP, gold nanoparticles; MWCT, multi-walled carbon nanotubes.

The analytical performance of the CHIT/GCE$_a$ electrode in real samples was tested performing the quantification of Trp in a commercial pharmaceutical supplement (Vitabay® VB 1057 500 mg). The measured Trp content of the commercial tablet prepared, as described in Section 2, was 520 (±50) mg in agreement with the certified content of 500 mg Trp of the commercial product. Finally, in order to estimate the recovery of the performance of the modified electrode, we evaluated the $i_{pa}$ for 25 μM Trp in presence of 30 μl of the diluted sample solution, and we compared it with the value obtained in absence of the sample. The recovery of the signal was 104%, pointing out that the electrochemical detection of Trp at CHIT/GCE is an accurate, precise and reproducible method [29].

## 4. Conclusions

In the present work, an electro-activated glassy carbon surface with adsorbed chitosan was prepared in a simple, facile and reproducible fashion. Complementary electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) experiments demonstrated that the electro-oxidation reaction of Trp on bare and CHIT-modified electrochemically activated GCE involves an irreversible two-electron and two-proton transfer process, and that the biopolymer is absorbed as a polycation at pH < 6.8, by protonation of the –$NH_2$ groups. The main effect of the adsorbed CHIT film is to produce a ≈4-fold increases of the oxidation current of Trp without changing either the oxidation potential or the heterogeneous reaction rate constant, suggesting that the biopolymer plays a role as a proton relay species, probably due to hydrogen bonding/proton acceptor capability of hydroxyl and ether groups present in CHIT.

Finally, differential pulse voltammetry (DPV) experiments proved that the CHIT/GCE$_a$ electrode shows a sub-micromolar detection limit for Trp with a linear range of two-order of magnitude, with almost no interference effect of other amino acids and good reproducibility. These promising analytical features of the modified CHIT/GCE$_a$ for electrochemical detection of Trp in acidic and neutral solutions can be applied for both qualitative and quantitative analysis of Trp in food and in pharmaceutical formulations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.11.087.

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