Voltammetric Determination of Indole-3-acetic Acid in Extract of *Pistia stratiotes*

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Abstract:
A simple, fast and direct method to determine indole-3-acetic acid (IAA) was developed using a carbon paste electrode (CPE) as working electrode and square wave voltammetry (SWV) as an electrochemical method. The experimental and instrumental parameters were optimized taking into account the values that provided the best performance in terms of peak current intensity of IAA, being the best values for: pH 1.8, Pulse amplitude (mV) 25, Step potential (mV) 12.5, Frequency (Hz) 80. After this, an analytical curve was obtained in the concentration range of 1 - 12 x 10⁻⁶ mol L⁻¹. From the analysis of the curve the limits of detection and quantification were determined, obtaining the following values, 45.73 x 10⁻⁹ mol L⁻¹ and 152.44 x 10⁻⁹ mol L⁻¹, respectively. Using the methodology developed, the amount of IAA present in the *P. stratiotes* extracts was determined. To eliminate possible interferences the standard addition method was performed, using 100 μL of *P. stratiotes* extract, fortified with different concentrations of the analytical standard of the IAA. Was found in average of 0.13±0.01 μg/g (IAA/plant) in the extracts (*n* = 2). The present investigation shows that the complex matrix does not interfere in the proposed method, reinforcing the applicability of the electrochemical sensors in real samples.

Keywords: cyclic voltammetry; square wave voltammetry; *Pistia stratiotes* extract; carbon paste electrode

1. Introduction
The indole-3-acetic acid (IAA) is a phytohormone of the auxin family, which acts as a chemical messenger, regulating stretching, differentiation and cell division. It is produced in the apical meristem of the stalk, leaf primordia, flowers, fruits and seeds, and it is transported throughout the plant by the conducting vessels xylem and phloem [1]. Although plants have low concentrations of IAA, it is responsible for controlling important physiological activities, such as initial development and differentiation in various parts of the plant body, including the differentiation of the driving elements in the leaves, gravitropism and apical dominance, directly linked to the growth and development of plants [2].

The determination of IAA in plants is of great significance to explore their biological roles, especially for understanding their physiological role. Moreover, measure the amount of this phytohormone can be an important parameter for the evaluation of plant stress, since its concentration can be affected by environmental injuries [3]. Commercially, the IAA is important due to the applicability of synthetic formulas of this phytohormone. In agriculture it is applied to control the growth of the crop and thus obtaining better yield, speeding up the cultivation process [4].

Determination of phytohormones in plant extract is generally quite difficult, due to their presence in minimal amounts, and the presence of a wide variety of interferents [5]. However, a variety of techniques have been employed for the detection of IAA, including chemiluminescence [6], liquid chromatography [7], gas chromatography [8], capillary electrophoresis [9], fluorescence spectroscopy [10], enzyme-linked immunosorbent assay [12], tandem mass spectrometry [11], immunoassay [12], and colorimetric detection [13]. However, there is still
interest in the development of increasingly simple, cheap, accurate and direct methods for the determination of IAA in plant extracts.

In this sense, electrochemistry is a good alternative for determining IAA, since the carbon-nitrogen bond of the heterocyclic existing in the Chemical structure of indole-3-acetic acid (Figure 1) makes possible its electrochemical determination. The nitrogen atom presents in the heterocyclic may be oxidized on the surface of the working electrode. The electrochemical oxidation of IAA can form the following products: 3-hydroxy-2-oxindole; indole-3-aldehyde; 3-methylene-2-oxindole; 3-(3′-methylene indole)-3 hydroxymethyl-2-oxindole and 2-(3′-methylene indole)-indole-3-acetic acid [14]. The electrochemical techniques, especially those of pulse (square wave voltammetry and differential pulse voltammetry) present high sensitivity, selectivity, repeatability, reproducibility, fast response, and low cost, making use simple appliances and easy to handle [1].

![Figure 1. Chemical structure of indole-3-acetic acid.](image)

The literature describes some electrodes used for the electrochemical determination of IAA in plant extracts and soil samples. In this sense, we can quote a carbon paste electrode modified with OV-17 silicone [15], a carbon fiber ultramicroelectrode [16], a carbon nanotube film modified glassy carbon electrode [17], a sodium dodecyl sulfate modified carbon paste electrode [18], a composite electrode of graphite and polyurethane [19], and a boron doped diamond electrode (BDD) [20]. The electrodes based on carbon have often been used for electrochemical determination, because their surfaces are easily renewed, have a low background current and a wide potential interval. These electrodes have low solubility in the working environment, elevated chemical purity of the reagents used to paste preparation, low adsorption of electroactive impurities and have a uniform granulometric distribution [21].

*Pistia stratiotes* was used to make the samples (extracts) analyzed in the present research, it is a plant popularly known as water lettuce, free floating aquatic herb, annual or perennial [22]. Endemic species of South America, although it is currently present in many parts of the world, especially in tropical and subtropical areas, where the environmental conditions allow its growth [23]. Temperatures around 15-35 °C and acid waters with neutral pH of 6.5 - 7.0 are favorable conditions for their growth [24]. *P. stratiotes* has been reported as a very efficient plant for the treatment of wastewater, is considered phytoremediate of contaminated environments, removing pollutants from different sources such as agriculture, industry, hospitals and household waste [25]. In addition, it has been reported in the literature as accumulator of trace metals such as Cr, Cu, Fe, Mn, Ni, Pb and Zn [26].

In the present paper, we used a carbon paste electrode (CPE), cyclic voltammetry (CV) and square wave voltammetry (SWV) for study the electrochemical behavior of IAA and to develop a new method through the optimization of instrumental parameters of the SWV (frequency, amplitude and potential step ) and experimental (pH) successfully applied to determine the IAA in real samples (*P. Stratiotes* extracts).

### 2. Results and Discussion

#### 2.1. Electrochemical behavior of IAA

The electrochemical behavior of IAA (280 x 10⁻⁶ mol L⁻¹) was investigated using CPE as a working electrode. The voltammogram obtained in the absence of the IAA did not show any peak in the range of evaluated potential, indicating that this region can be used to investigate the redox process of the proposed molecule (voltammogram a, black, Figure 2). In the presence of the IAA, the voltammograms obtained for the first potential scan in the anodic sense (0: 1.25 V) and for reverse scan (1.25: 0 V), presented two oxidation peaks (anodic peak), Iₐ and IIₐ, with peak potential (Ep) of 870 mV and 1030 mV, respectively, and a reduction peak (cathodic peak), IVₒ in the reverse scan Ep = 386
mV, (voltammogram b, red, Figure 2). The second successive scan in the same potential range showed in the voltammogram two anodic peaks, \( V_A \) (\( E_p = 500 \) mV) and \( \text{III}_A \) (\( E_p = 1000 \) mV), and a cathodic peak \( IV_C \) (\( E_p = 380 \) mV) (voltammogram c, blue, Figure 2). The results obtained and presented in Figure 2 are similar to the results obtained when using silicone OV-17 modified carbon paste electrode [27] and pyrolytic graphite electrode as work electrode [14].

**Figure 2.** Cyclic Voltammetry of IAA (280 x 10^{-6} mol L^{-1}) using a CPE. Support electrolyte of KCl/HCl (0.2 mol L^{-1}), pH = 1.80. Potential range: 0:1.25:0 V. \( \nu = 0.1 \) V s^{-1}.

The voltammetric profile is heavily influenced by IAA concentration present in the electrochemical cell (Figure 3, Table 1). The voltammograms obtained for the first and second scanning in a solution containing 7.15 x 10^{-6} mol L^{-1} of IAA are shown in Figure 3A. where we observed in the first scan, in the anodic direction (black voltammogram, Figure 3A), only one oxidation peak (\( \text{III}_A \)) and in the second successive scan (blue voltammogram, Figure 3A), two anodic peaks (\( IV_A \) and \( \text{III}_A \)). Considering the cathodic direction of the scanning potential, we observed only a reduction peak (\( IV_C \)) in the first and second successive scan for all concentrations tested. The increase of the IAA concentration in the electrochemical cell to 119.55 x 10^{-6} mol L^{-1} (Figure 3B) and later to 228.48 x 10^{-6} mol L^{-1} (Figure 3C), significantly altered the redox process of the IAA on the CPE surface, affecting the voltammetric profile. In the two highest concentrations tested, two anode peaks (\( IA \) and \( IIA \)) appeared. The values obtained for \( E_p \) (mV)/ Ip (\( \mu A \)) were: 870/3.14 (\( I_A \), scan 1), 1024/4.65 (\( IIA \), scan 1), and 870/8.62 (\( I_A \), scan 1), 1020/13.95 (\( IIA \), scan 1) for 119.55 x 10^{-6} mol L^{-1} and 228.48 x 10^{-6} mol L^{-1}, respectively. The decrease observed at the Ip values, when the comparison is performed between the first and successive second scan of the potential, can be attributed to IAA adsorption on the surface of the working electrode. Similar results are described in the literature [14]. Regarding the cathodic processes that occur on the CPE surface, no significant differences were observed (Figure 3).

**Figure 3.** CVs in different concentrations of IAA using a CPE. 7.15 x 10^{-6} mol L^{-1} (A), 119.55 x 10^{-6} mol L^{-1} (B), and 228.48 x 10^{-6} mol L^{-1} (C). Experimental conditions: see material and methods. pH = 1.80. \( \nu = 0.1 \) V s^{-1}.
### Table 1. Concentration: A = 7.15 x 10^{-6} mol L^{-1}, B = 119.55 x 10^{-6} mol L^{-1}, C = 228.48 x 10^{-6} mol L^{-1}.

| Concentration | Scan | Peaks | \(I_p\) (\(\mu A\)) | \(E_p\) (mV) |
|---------------|------|-------|----------------|-------------|
| A             | 1st  | I_A   | --             | --          |
|               |      | II_A  | --             | --          |
|               |      | III_A | 4.45           | 940         |
|               |      | IV_A  | --             | --          |
|               |      | IV_C  | -0.11          | 411         |
|               | 2nd  | I_A   | --             | --          |
|               |      | II_A  | --             | --          |
|               |      | III_A | 2.56           | 943         |
|               |      | IV_A  | 0.12           | 433         |
|               |      | IV_C  | -0.10          | 413         |
| B             | 1st  | I_A   | 3.14           | 870         |
|               |      | II_A  | 4.65           | 1024        |
|               |      | III_A | --             | --          |
|               |      | IV_A  | --             | --          |
|               |      | IV_C  | -0.43          | 379         |
|               | 2nd  | I_A   | --             | --          |
|               |      | II_A  | --             | --          |
|               |      | III_A | 3.14           | 1010        |
|               |      | IV_A  | 0.24           | 502         |
|               |      | IV_C  | -0.25          | 362         |
| C             | 1st  | I_A   | 8.70           | 870         |
|               |      | II_A  | 13.95          | 1020        |
|               |      | III_A | --             | --          |
|               |      | IV_A  | --             | --          |
|               |      | IV_C  | -0.20          | 375         |
|               | 2nd  | I_A   | --             | --          |
|               |      | II_A  | --             | --          |
|               |      | III_A | 4.65           | 1040        |
|               |      | IV_A  | 0.20           | 505         |
|               |      | IV_C  | -0.20          | 375         |

(--*) absent. (***) Difficult to determine \(E_p/I_p\) values (low values).

#### 2.2. Effects of pH

The results obtained in this study are presented in Figure 4. As pH increased, the \(E_p\) values change linearly to more negative values (Fig. 4B), indicating the intervention of protons in the electrochemical oxidation of IAA on the surface of the working electrode. The values obtained were adjusted using the least squares method, yielding the following equation: \(E_p\) (mV) = 926.45 - 34 \(\Delta\) pH, \(r = 0.99\). The value obtained for the angular coefficient of the curve \(E_p\) vs. pH were 34.15 mV/\(\Delta\)pH, suggesting that different amounts of electrons and protons are involved in the IAA oxidation the surface of the CPE [14]. In this case, the amount of electrons is double the amount of protons. This result is in agreement with results described in the literature, that is, 2 electrons and 1 proton are involved in the electrochemical oxidation of the IAA [14]. A maximum \(I_p\) value was recorded at pH 1.80 (Figure 4C), this pH was selected for the electrochemical study of IAA and for development of an electrochemical method for determination of IAA in the studied samples.

![Figure 4](image)

2.3. Optimization of parameters: SWV

The instrumental parameters optimized were: frequency, potential step and amplitude of pulse application. The frequency is an important...
parameter of SWV, since it can increase the sensitivity of the developed method, resulting in a higher Ip value of the electrochemical reaction that occurs on the working electrode surface [29]. The Ip values in function of frequency variation were determined from the voltammograms obtained for a solution containing $9.95 \times 10^{-6}$ mol L$^{-1}$ of IAA in the electrochemical cell (voltammograms not shown). It is observed a linear increase in the values of Ip with the increase of the frequency up to 80 s$^{-1}$ according to the equation $Ip (\mu A) = 1.49 + 0.10 f (s^{-1}), r = 0.99$. After this value, it was observed a decrease in the Ip values for higher frequencies (Figure 5A). This decrease is probably related to the saturation on the CPE surface. The linear increase of Ip with the increase of the frequency application of pulses is characteristic of irreversible electrochemical reaction on the surface of the working electrode [29]. Thus, taking into account all the mentioned aspects, the frequency value of 80 s$^{-1}$ was selected for later studies.

![Figure 5](image-url)

**Figure 5.** Optimization of parameters SWV. A) Ip vs f. B) Ep vs log f. C) Ip vs Step. A) Ip vs A.

Experimental conditions: Support electrolyte of KCl/HCl (0.2 mol L$^{-1}$), pH = 1.80. Potential range: 0:1.25:0 V. $v = 0.1$ V s$^{-1}$. [IAA] = 9.95 x 10$^{-6}$ mol L$^{-1}$. (Five replicates: n = 5).

In relation to the Ep values, a displacement to more positive values is observed, when the frequency is increased (Figure 5B), this characteristic may mean that the presence of protons may interfere with the electrochemical reaction that occurs on the surface of the working electrode. The amount of electrons involved in the oxidation reaction of the IAA on the CPE surface was estimated using the following equation: $slope = 2.3 \frac{RT}{\alpha nF}$, slope is the angular coefficient of the straight $dE_p/d\log f$, $R = \text{universal gas constant}$, $T$ is the temperature in K, $F$ is Faraday's constant, $\alpha$ is the load transfer coefficient (0.5 for an irreversible reaction), and $n$ is the amount of electrons involved in the reaction. The slope determined from Figure 5B was 53.02 mV, this value was used to determine the number of electrons, $n$, and the value found was 2.2, indicating the participation of 2 electrons in the oxidation of IAA on the CPE surface. This result is in agreement with the amount described in the literature [14].

In relation to the potential step, it was sought a value that, the peak current was higher without loss of symmetry. This care is necessary because peaks with loss of symmetry, even with higher current values present an increase in W1/2 (half-
wave potential), which results in the increase of the peak area and consequently the peak loses resolution, selectivity and accuracy. Therefore, scans were performed in a range from 5 to 20 mV (Figure 5C), and the potential step value of 12.5 mV was selected for further studies.

For the amplitude, the scans were performed in a range of 10 to 100 mV (Figure 5D). By varying this parameter peak current intensity (greater sensitivity) is observed for higher amplitudes, however there is a deformation of the peaks, which results in the loss of the selectivity. Thus, it was decided to fix the amplitude in 25 mV. Table 2 summarizes the SWV parameters and the pH value that presented the best performance in relation to Ip, for the instrumental and experimental parameters investigated in this study.

### Table 2. Optimal parameters in SWV and optimal pH. KCl/HCl buffer: 0.2 mol L⁻¹ pH = 1.80.

| Parameters        | Range (mol L⁻¹) | Optimized value |
|-------------------|-----------------|-----------------|
| pH                | 1.0 - 2.2       | 1.8             |
| Pulse amplitude (mV) | 10 – 100       | 25              |
| Step potential (mV) | 5 – 20         | 12.5            |
| Frequency (Hz)    | 5 – 110         | 80              |

**2.4. Limit of quantification and limit of detection**

The analytical curve was obtained using the CPE, and the values of frequency, amplitude, step potential, and pH optimized in terms of peak current intensity of IAA oxidation (Table 3). The electrochemical method used was the SWV due to its high sensitivity.

The Ip values showed a linear increase with the increase concentration. Table 3 shows the concentration range, LD, LQ and sensitivity, determined from the analytical curve obtained. The analytical curve equation was calculated through the least square’s method, as follows: \( Ip (\mu A) = -0.152\pm0.005 + 0.328\pm0.002 [IAA] \times 10^{-6} \) mol L⁻¹) \( (R = 0.999) \). Limits of quantification (LQ) and detection (LD) were determined using the relationships LD = 3SD/s and LQ = 10SD/s; where: “s” is the analytical curve slope and SD the standard deviation of the intercept according to IUPAC recommendations [30]. Table 4 shows a comparison of the methodology proposed in the present research with other methodologies described in the literature. Despite the similarities in performance between this and other studies, the ease of use, speed, cost and sensitivity of the proposed method make it possible to determine the IAA in real samples.

### Table 3. Parameters obtained from the analytical curve employing SWV.

| Parameters        | Values |
|-------------------|--------|
| Range (10⁻⁶ mol L⁻¹) | 1 - 12 |
| Intercepts (µA)    | -0.152±0.005 |
| Slope (µA/10⁻⁶ mol L⁻¹) | 0.0328±0.002 |
| LD (10⁻⁹ mol L⁻¹)  | 45.73  |
| LQ (10⁻⁹ mol L⁻¹)  | 152.44 |

**2.5. Determining IAA in plant extracts**

The quantities of IAA present in each sample were determined using the proposed methodology and standard addition method to minimize the effect of interferents present in the P. stratiotes extracts. For the extract 1 (0.12 µg/g) of IAA was found, while, in the extract 2 was found (0.14 µg/g) of IAA, the average of the two replicas is 0.13 µg/g of IAA with SD 0.01. The quantification of phytohormone in plant sample is a procedure that requires a sensitive and selective methodology, since the plants have a great variety of compounds in small quantities [26]. However, the methodology developed was successfully applied for IAA determination in the investigated sample.
**Table 4.** Studies reporting IAA quantification using electrochemical methods.

| Modifier/Electrode | Method  | Linear range (mol L\(^{-1}\)) | LD (mol L\(^{-1}\)) | Samples analyzed                                         | Ref.   |
|-------------------|---------|--------------------------------|----------------------|----------------------------------------------------------|--------|
| GCE               | DPV     | 1.8 × 10\(^{-6}\) – 6.6 × 10\(^{-4}\) | 2.7 × 10\(^{-6}\)   | *Nicotiana Langsdorffii*                                  | [31]   |
| BDD               | SWV     | 5.0 × 10\(^{-6}\) – 5.0 × 10\(^{-5}\) | 1.2 × 10\(^{-6}\)   | *Peganum harmala L.*, *Triticum aestivum L.*, *Peganum harmala L.*  | [19]   |
| GR-P/MWNT         | DPV     | 1.0 × 10\(^{-7}\) – 7.0 × 10\(^{-5}\) | 5.0 × 10\(^{-8}\)   | *Cinnamomum camphora*, *Prunus yedoensis*                  | [1]    |
| GR/Pol            | SWV     | 5.5 × 10\(^{-6}\) – 8.5 × 10\(^{-5}\) | 1.1 × 10\(^{-7}\)   | Soil                                                      | [17]   |
| CPE/OV-17         | DPV     | 4.2 × 10\(^{-7}\) – 2.5 × 10\(^{-6}\) | 225.2 × 10\(^{-9}\) | *Ecballium elaterium* (L) stems                           | [14]   |
| CPE               | SWV     | 1.0 × 10\(^{-6}\) – 1.2 × 10\(^{-5}\) | 45.73 × 10\(^{-9}\) | *Pistia stratiotes*                                        | This work |

GCE: Glassy carbon electrode. DPV: Differential pulse voltammetry. SWV: square wave voltammetry. OV-17: Silicone OV-17. GR/Pol: graphite-polyurethane GR-P/MWNT: Graphite pencil/Multiwalled carbon nanotubes (MWNTs). BDD: Boron-doped diamond electrode.
3. Material and Methods

3.1. Reagents and solutions

All chemicals used are analytical grade, the indole-3-acetic acid (Sigma-98%), methyl alcohol (Vetec 96%), ethyl alcohol (Neon 95%), ethyl ether (Qhemis 95%), graphite Sigma-Aldrich), particle size <20 μm) and nujol (Sigma-Aldrich). Aqueous solutions were prepared using distilled water. The stock solution of IAA (7.152 x 10^{-3} mol/L) was prepared with ethyl alcohol, from this, a working solution in 0.2 M HCl, pH 1.8 was made. A phosphate buffer solution (0.2 mol L^{-1} KCl/HCl) was used as support electrolyte.

3.2. Apparatus

All CV and SWV measurements were performed on an Autolab PGSTAT12 instrument (Eco Chemie, Utrecht, The Netherlands), coupled to a computer with the NOVA program 1.10. In an electrochemical cell with a capacity of 30 mL, the 0.2 mol L^{-1} KCl/HCl buffer was stored, and the anolyte in the concentrations applicable to each phase of the research. In addition to the working electrode, two auxiliary electrodes were used, platinum wire as the counter-electrode, and Ag/AgCl in KCl (3 mol L^{-1}) as the reference electrode. During the measurements the electrochemical cell was placed in a Faraday cage to minimize background noise.

3.3. CPE preparation

The preparation of the carbon paste electrode (CPE) consists of a mixture of graphite (Sigma-Aldrich, particle diameter < 20 μm) and nujol mineral oil (Sigma-Aldrich) in a proportion of 70% graphite and 30% nujol. The paste was obtained by macerating the graphite with nujol for 40 minutes in a glass crucible. The prepared paste was placed in a 1.0 mL plastic syringe; the electrical contact was established by a copper wire inserted into the electrode. The renewal of the surface of the working electrode was performed using a sheet of A4 paper.

3.4. Parameter optimization

For all optimization of experimental and instrumental parameters, square wave voltammetry (SWV) and CPE as the working electrode was used, and in the electrochemical cell 5 mL of 0.2 mol L^{-1} KCl/HCl buffer. Initially, the effect of pH on the electrochemical behavior of the IAA was evaluated, in the pH range from 1.00 to 2.20 and an IAA concentration of 3.56 x 10^{-6} mol L^{-1}. Then, the SWV parameters, amplitude (A), frequency (f), potential step (s) (IAA concentration of 9.95 x 10^{-6} mol L^{-1}). This optimization was done from the evaluation of the Ip and Ep of each parameter studied. It is important to highlight that standard deviation (SD) values were always obtained from the average of 5 replicates.

3.5. Sample preparation: extracts of *Pistia stratiotes*

The plants used in this study were collected in the Pantanal Sul Mato-Grossense and were aclimatized in a greenhouse. The plant extracts (in duplicate) were made with fresh mass of P. stratiotes ramets (young plant identical to the mother plant, originated by sexual reproduction, using 50.248 g for replica 1, and 50.754 g for replica 2. Thus, in a porcelain mortar with the aid of a pistil the vegetable mass was macerated with 100 ml of methanol added gradually. The extract was filtered using vacuum pump and filter paper then was adjusted to pH 10 using NaOH 0.2 M. Then the IAA extraction process was started, based on the methodology of Hernández et al. (1996) [28]. The filtrate was mixed with 50 mL of diethyl ether and then taken to a 250 mL separatory funnel. This mixture was shaken manually for 5 minutes, afterwards it was waited until 2 phases were formed, then the supernatant was collected, and the precipitate was filtered 2 more times in 50 mL of diethyl ether. After this procedure all the supernatant was adjusted to pH 2 using HCl 0.2 M, then the phase separation process described above was carried out for a further 3 times. The supernatant collected in this last stage of the IAA extraction process was placed in petri dish and taken to the ductless fume hoods, where it was maintained until complete evaporation of the extractive solvent. The residue was then recovered in 10 mL of absolute ethanol.

4. Conclusion

The results obtained show the efficacy of the carbon paste electrode and square wave voltammetry for quantification of the IAA
phytohormone in samples of P. stratiotes extract. This shows the sensitivity and applicability of the electrochemical sensors in real samples, since the plants have low amount of IAA, among a wide variety of interferents. The quantification of this hormone in plant samples has great importance, therefore it is a strong parameter to evaluate plant stress, once the plants can alter the production of this hormone as a result of disturbances caused by the environment.

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