Determination of indole-3-acetic acid and indole-3-butyric acid using HPLC-ED with carbon felt as a working electrode

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ABSTRACT

Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are a class of phytohormones known as auxins. These indoles are crucial in plant development and growth. They are commonly commercialized in agricultural products and their proper quantification is essential since their excess can cause the opposite effect in the plants or even be prejudicial to public health. However, their determination is difficult due to their instability. Thus, it is recommended to use a sensitive, simple, and accurate method for their detection. Among the existing techniques for these auxins' quantification, the high-performance liquid chromatography (HPLC) is highlighted, in which several types of detectors can be used for the determination of IAA and IBA. Then, as it is possible to obtain a good quantification of them through electrochemical methods, the proposed alternative in this present work is the utilization of carbon felt (CF) as a working electrode at the amperometric detector attached to HPLC (HPLC-ED) for the simultaneous determination of these phytohormones in gardening products samples. The CF shows advantages such as good electric conductivity and high chemical, physical and mechanical stability; besides, it is a cheap manufactured material. In order to reach the best parameters to optimize the results, blank solutions were used in the UV-vis spectrophotometry to achieve absorbance and a stock solution with a known concentration was used in the HPLC to achieve the percentage of methanol, pH and potential for the mobile phase. The found values were: wavelength of 220 nm, 60% of methanol, pH 4.0, and potential +1.5 V. The applicability of this method was confirmed by verifying the concentration linearity; the obtained quantification limit (LOQ) for IAA and IBA was 1.1 μmol dm^{-3} and 1.8 μmol dm^{-3}, respectively. Therefore, the proposed method was efficient for the quantification of the auxins in the tested product. It was compared with an already consolidated method, HPLC with UV spectrometric detection (HPLC-UV), which was used as reference method. The percentage results of these auxins in the gardening sample, Stimulax ll, for both methods agreed between them, proving the applicability of HPLC-ED for these types of samples.

Keywords: *Auxins; Carbon felt; Electrochemistry; High-pressure liquid chromatography.*

RESUMO

Ácido indol-3-acético (AIA) e ácido indol-3-butírico (AIB) são conhecidos como auxinas, uma classe de fito-hormônios. Essas indols têm papel importante no desenvolvimento e crescimento das plantas. Eles são comumente comercializados em produtos agrícolas, sendo de grande importância sua quantificação adequada, uma vez que sua aplicação em excesso pode causar o efeito contrário do desejado nas plantas e/ou até mesmo ser prejudicial à saúde pública. Estes componentes são de difícil determinação devido sua instabilidade, portanto é recomendado a utilização de um método sensível, simples e preciso para a sua detecção. Entre as técnicas existentes para quantificação dessas auxinas é destacada a cromatografia líquida de alta eficiência (HPLC), na qual pode ser utilizado diferentes tipos de detectores para a determinação do AIA e AIB. Assim, como é possível obter uma boa quantificação dessas auxinas através de técnicas eletroquímica, a alternativa proposta no presente trabalho é utilização de feltro de carbono como eletrodo de trabalho no detector amperométrico acoplado no HPLC (HPLC-ED) para determinação simultânea desses fito-hormônios em amostras de produtos de jardinagem. O feltro de carbono, apresenta vantagens como boa condutividade, alta estabilidade química, mecânica e física além de ser um material fabricado a baixo custo. A fim de atingir os melhores parâmetros para otimizar os resultados, soluções padrão branco foram utilizadas no espectrofotômetro UV-Visível para descobrir a absorbância e uma solução padrão com concentração conhecida foi usada no HPLC para encontrar a percentagem de metanol, pH e potencial para a fase móvel. Os valores obtidos foram: 220 nm de comprimento de onda, 60% de metanol, pH 4,0, e potencial de +1,5 V. A aplicabilidade desse método foi confirmada verificando a linearidade da concentração e obtendo os valores dos Limites de Quantificação (LOQ) para AIA e AIB da amostra: 1,1 µmol dm^{-3} e 1,8 µmol dm^{-3}, respectivamente. Portanto, o método proposto foi eficiente para realizar a quantificação dessas duas auxinas no produto testado, visto que foi comparado com um método já consolidado, HPLC com ultravioleta-visível como detector (HPLC-UV), que foi utilizado como método de referência. Os resultados das porcentagens dos compostos presentes na amostra do produto de jardinagem, Stimulax ll, para os dois métodos estavam de acordo, comprovando a possibilidade de aplicação do HPLC-ED para esses tipos de amostras.

Palavras-chave: *Auxinas; Feltro de carbono; Eletroquímica; Cromatografia líquida de alta eficiência.*

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SUMMARY

1 Introduction

Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are a class of phytohormones known as auxins. They are produced by plants themselves and are essential for controlling and regulating development processes, such as growth, germination, division and differentiation of cells. They are commonly commercialized in agriculture products for plant rooting stimulators or pesticides. They used in excess can cause the opposite effect in the plants or even be prejudicial to public health. Therefore, it is crucial to develop a precise and simple method to determine and quantify as precisely as possible their trace in plants samples to avoid their excess or as an attempt to understand even more their process in plants (HAN *et al.*, 2012; SUN *et al.*, 2014).

As the concentration determination of these auxins is difficult since their instability, it is recommended a sensitive and accurate method for their detection, as a quantitative analytical. In the literature exists several works presenting techniques for the auxin quantification. The most common techniques are high-performance liquid chromatography (HPLC) and gas chromatography (GC). Studies have already been reported on the determination of these phytohormones using HPLC with different detectors, such as UV spectrometric detection (HPLC-UV); thin-layer chromatography (HPTLC) and tandem mass spectrometry (HPLC–MS/MS).

As these indoles compounds allow their quantification through electrochemical methods in virtue of their electrochemical oxidizability, a cheap, fast, simple, and accurate alternative for their determination is to use carbon felt (CF) as a working electrode at the amperometric detector equipment for HPLC. This material exhibits advantages such as good electric conductivity and high physical, mechanical and chemical stability. Their use in analytical electrochemistry or environmental applications and energy purposes is growing since they are an inexpensive manufactured material.

The main objective of this study is to develop a new detection method for simultaneous determination of the phytohormones IAA and IBA in gardening products samples, as a quantification alternative. The specific objectives are to study and review literature methods for this determination; optimize the bests parameters to set in the apparatus to achieve the best results such as absorbance, percentage of methanol and pH in the mobile phase and potential; validate the stability, precision and applicability of the method through concentration dependence comparing with a reference method (HPLC-UV); confirm the values agreement of the two methods with the displayed on the label of the gardening product, Stimulax II.

The following work was accomplished at the beginning of 2018 during a 3 months volunteer internship exchange in Prague, Czech Republic. The research was

carried out at the United Nations Educational, Scientific and Cultural Organization (UNESCO) Laboratory of Environmental Electrochemistry located at Charles University under the supervision of RNDr. Hana Dejmková, Ph.D.

2   Literature Review

In this chapter, the theoretical foundations that supported the undergraduate dissertation will be described. It will introduce an overview of the indole compounds. Moreover, it will approach some basic concepts to facilitate the understanding of the electrochemical analysis, the difference between the electrode's types, highlighting the carbon felt, ultraviolet spectrophotometry and chromatography process, as HPLC. As well, works found in the literature for the auxin quantification methods will be presented.

2.1   Basic and Theoretical Foundations

2.1.1 Compounds

Indole-3-acetic acid (IAA, Figure 1a) and indole-3-butyric acid (IBA, Figure 1b) are auxins that are a class of phytohormones produced by plants themselves and important to the development of plants, which exert and control physiological processes and are essential to regulation of germination, growth, reproduction, organ formation, seed dormancy and protective responses of plants against stress. Also, these auxins regulate processes such as inducing, elongation, division and differentiation of cells. Plant hormones are typically present and active in minor concentrations in tissues (HAN *et al.*, 2012; SUN *et al.*, 2014).

However, light, oxygen, and heat effortlessly decompose these auxins (LU *et al.*, 2016). Both of these phytohormones are ingredients in many commercial plant rooting stimulators in horticultural products and pesticides in agriculture products (DEJMKOVA; DANIEL, 2019). The main properties of these two indole auxins are shown in Table 1.

Figure 1 - Chemical structures of the auxins: (a) indole-3-acetic acid; (b) indole-3-butyric acid.
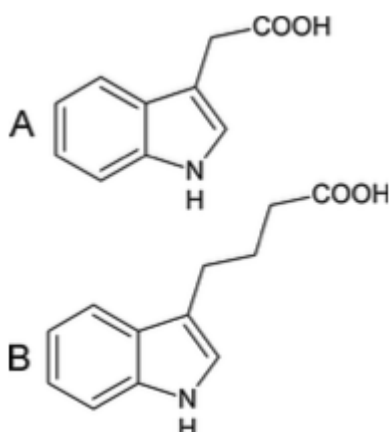
Source: (DEJMKOVA; DANIEL, 2019)**.**

Table 1 - Properties of indole-3-acetic acid and indole-3-butyric acid.

	IAA	IBA
Name (IUPAC)	2-(1H-indol-3-yl)acetic acid	4-(1H-indol-3-yl)butanoic acid
Common Name	Indole-3-acetic acid	Indole-3-butyric acid
Molecular Formula	$C_{10}H_9NO_2$	$C_{12}H_{13}NO_2$
Molecular Weight	175.18	203.24
CAS Number	87-51-4	133-32-4
Melting Point [°C]	168.5	124.5

Source: Elaborated by the author based on PubChem.

2.1.2 Electrochemistry

The participating materials must have electric conduction (free electrical charges) as an intrinsic characteristic. This conduction can be electronic (metals, organic or inorganic substances) or ionic (called electrolytes) and it is classified based on the type of the transported charge in the electric current. Thus, the electrochemical system must be composed of at least two electrical conductors (cathode and anode electrodes) immersed in an electrolyte that transports ions (TICIANELLI; GONZALEZ, 2005; SMITH; STEVENSON, 2010; GALLO, 2019).

The fundamental goal of electrochemical is to study systems capable of producing electrical energy from the electron migration from the anode (oxidation reaction) to the cathode (reduction reaction). These systems can be spontaneous oxidation-reduction reactions (galvanic cells), which the cathode is the positive pole

and the anode is the negative pole. On the other hand, the non-spontaneous reaction is called electrolysis and the cathode is the negative pole and the anode is the positive pole. These two cases are schematic in Figure 2 (TICIANELLI; GONZALEZ, 2005; SMITH; STEVENSON, 2010; GALLO, 2019).

Figure 2 - Schematic of common galvanic and electrolytic cells.
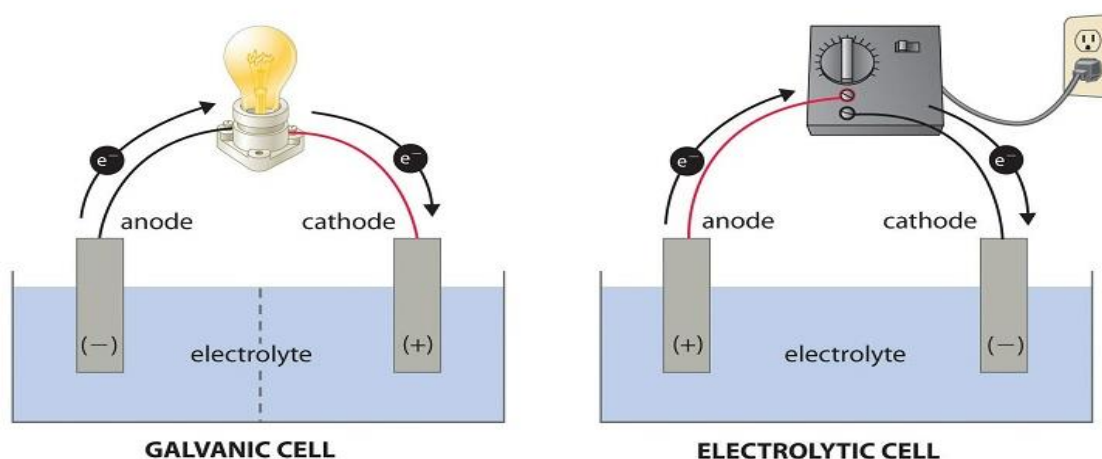
Source: Chemistry LibreTexts' page. (Available in: <https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Electrochemistry/Electrolytic_Cells>. Accessed on: April 20, 2022).

2.1.2.1 Electrochemical Analysis

Electrochemical analysis is used for environmental, biomedical and industrial applications (mostly in quality control). These technologies/techniques make it possible to quantify the potential, current and charge of chemical compounds. They must have an electrochemical cell with an electrolyte solution in contact with at least 2 electrodes (working and reference electrodes). There are diverse techniques such as voltammetry, amperometry and potentiometry, among others that apply this technology (WANG, 2006).

2.1.2.1.1 Voltammetry

Voltammetry is an electrochemical technique that shows qualitative and quantitative information about a chemical species by current-potential graphics (voltammograms). They are obtained from the analyte electrolysis in an electrochemical cell with at least two electrodes (working and reference electrode). Varying the potential in a constant velocity along time between these two electrodes, the potential and current can be registered simultaneously (ALEIXO, 2003).

2.1.2.1.2 Amperometry

Amperometry is an analytical technique used for pharmaceutical, environmental, agricultural and industrial applications. Their use as detector sensors is growing, since they can be used together with flow analysis through the application of constant potential (reducing or oxidizing) to a working electrode (WE) and the measurement of the current (proportional to the concentration of the analyte) in the function of time between at least a pair of electrodes (AMINE; MOHAMMADI, 2019).

This technique has some advantages, for example for electroactive species it has superior selectivity and sensitivity, cheap instrumentation, concentration linearity and several kinds of electrodes that allow unusual environmental analyses (AMINE; MOHAMMADI, 2019).

Besides, in combination with a flow analysis such as HPLC, the mobile phase passes through the flow-cell that WE is placed at, and the applied potential submits the analyte to a faradaic reaction. Joining these two methods, the detection of simultaneous analytes in complex analyses becomes possible (AMINE; MOHAMMADI, 2019).

2.1.2.2 <u>Electrodes</u>

2.1.2.2.1 Working Electrode

The working electrode (WE) is the one in contact with the analyte and where the important reaction occurs. For that reason, its potential is controlled. The main materials to manufacture them are carbon, mercury, or noble metals (such as gold and platinum). To determine the working electrode, it must consider the redox reaction in the analyte, background current, electrical conductivity, mechanical properties, availability and cost (WANG, 2006).

The use of carbon felt as an electrode material in analytical electrochemistry is growing, since this material shows high electric conductivity, large specific surface area, high porosity allowing to provide redox reaction sites and high physical, mechanical and chemical stability. This material is formed by randomly ordered carbon fibers. It is commercially available since it is industrially manufactured at a low price. In Figure 3, it is possible to see a typical commercial shape. They are used in many fields of science, for example electroanalytical (coulometry or anodic stripping voltammetry), energy purposes or environmental applications (like wastewater treatment) (GONZÁLEZ-GARCÍA *et al.*, 1999; HUONG LE; BECHELANY; CRETIN, 2017; (BAROCH; SLÁDKOVÁ, 2020).

On the other hand, the CF has some disadvantages: its structural geometry has not a simple symmetry (cylinder-loe shapes fibers) (GONZÁLEZ-GARCÍA *et al.*, 1999). Since they show hydrophobic surface and bad kinetics for oxidation and reduction

reactions, they have some disability for electrochemical activity in aqueous solutions and poor wettability. Decreasing their performance when applied to electrodes (HUONG LE; BECHELANY; CRETIN, 2017). Also, this kind of porous flow-through electrode shows difficulty when controlling the potential that is applied on the electrode. Since the potential drop in the electrode volume is prejudiced, causing on the contrary sides of this electrode distinct current efficiencies. Nevertheless, the advantages of carbon felt are much more relevant, especially their low price and their good electric conductivity (BAROCH; SLÁDKOVÁ, 2020; DEJMKOVA; DANIEL, 2019).

Figure 3 - Commercially shape of Carbon Felt.

Source: (HUONG LE; BECHELANY; CRETIN, 2017)**.**

2.1.2.2.2 Reference Electrode

The reference electrode (RE) is composed of a redox couple, usually Ag/AgCl or Hg/Hg_2Cl_2. Its purpose is to keep the potential constant and stable to be able to measure the potential change of the working electrode during the experiment, providing a reproducible potential (SMITH; STEVENSON, 2010; WANG, 2006).

2.1.2.2.3 Auxiliary/Counter Electrode

The auxiliary electrode (CE) is used in three-electrode cells and must be produced from an inert material, usually platinum, titanium or carbon. This electrode is used to complete the circuit to allow the charge to move. It is used as an intermediate bridge to avoid contact and contamination between the RE and the sample solution. It is also used to prevent the excess of current to arrive in the RE (SMITH; STEVENSON, 2010; WANG, 2006).
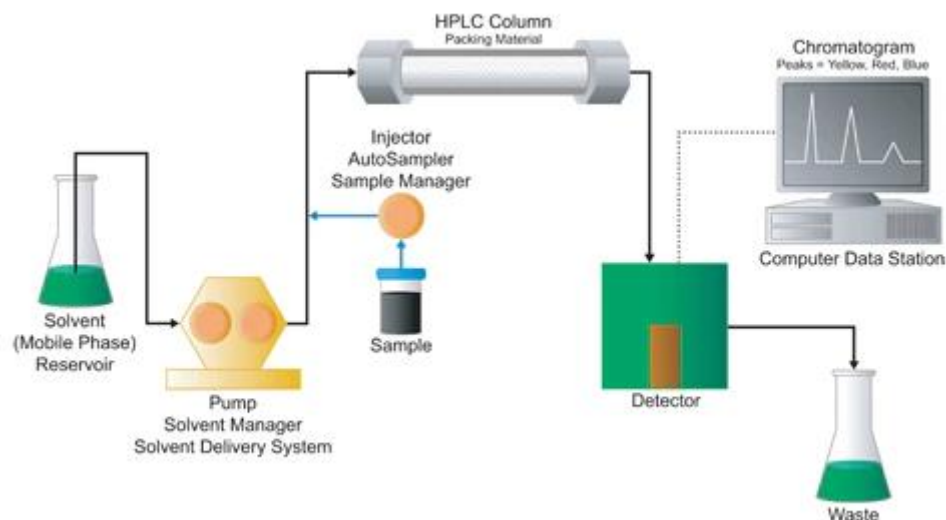
2.1.3   Chromatography

Chromatography is a physical process used to separate mixtures based on their compounds distribution between two phases, the mobile (liquid, gas or supercritical fluid, that percolates in a definite direction) and the stationary (liquid, gel or solid) phase. The aim of this technique is the analysis or future use of these samples (SANJEEVI; PANDEY, 2019).

The separation relies on the interaction of the sample components with the stationary phase, since they can be retained through the adsorption or partition, and also the velocity in which they are carried away by the mobile phase, since these mixtures pass across the stationary phase with the mobile phase. There are several classifications of the chromatographic techniques, like the ones based on phase (gas, liquid or supercritical-fluid chromatography), shape (column or planar chromatography), application (frontal, displacement or elution chromatography), among others (SANJEEVI; PANDEY, 2019).

Highlighting the high-performance liquid chromatography (HPLC), it is an analytical method more than 40 years old and it is used to separate, identify, quantify and purify components of liquid samples. It is possible to use it for the quantitative and qualitative analysis and it can be applied in many areas like in pharmaceutical industries, water purification, food analysis, environmental samples and others (SNYDER; KIRKLAND; GLAJCH, 1997; THOMAS, 2013).

A highly purified sample in a small amount is to be injected in the HPLC. Then, a high-pressure pump delivers the mobile phase at a controlled flow rate and it carries the injected sample to the HPLC column. Inside the column, the stationary phase interacts with the analytes. Components with stronger affinity with the mobile phase, moves through the column faster than the ones with higher affinity with the stationary phase. Therefore, this affinity is responsible for the separation of the target compounds from the sample. The detector is placed downstream of the column to identify and quantify the compounds as they eluate from the HPLC column. The chromatograms obtained from the detector can be analyzed at a workstation. This system is shown in Figure 4 (GOSWAMI; THAKKER; DHANDHUKIA, 2015; SUNIL, 2018; THOMAS, 2013).

Figure 4 - High-Performance Liquid Chromatography [HPLC] System.

Source: Waters' page. (Available in: <https://www.waters.com/waters/pt_BR/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?locale=pt_BR&cid=10049055>. Accessed on: April 15, 2022).

Thus, the sample velocity inside the column depends on several factors such as the nature of the column and the sample and the composition of the mobile phase. In Figure 5, it is possible to notice the analyte retention time as the separation occurs along the column and their detection time (SUNIL, 2018; THOMAS, 2013).

Figure 5 - Example of HPLC column separation and detection.
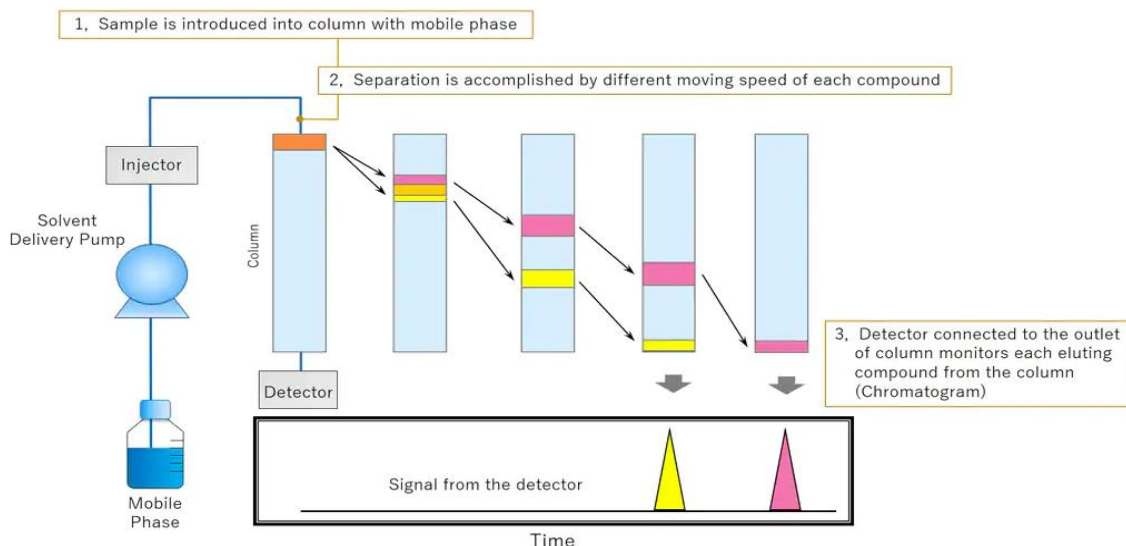
Source: Shimadzu's page. (Available in: <https://www.shimadzu.com/an/service-support/technical-support/analysis-basics/basic/what_is_hplc.html>. Accessed on: April 15, 2022).

There is a variety of detectors to use with HPLC, like ultraviolet/visible, electrochemical, and electrical conductivity, among others. However, all of them must show some common features such as linear analyte concentration response, reproducible and stable signals and non-interference of the variation of temperature (SUNIL, 2018).

HPLC has several benefits like broad applicability, high sensitivity, and good repeatability, since it is an automatic, easy to use, quick, efficient and affordable technique already "mature" as it has been practiced for about five decades (SNYDER; KIRKLAND; GLAJCH, 1997; THOMAS, 2013). In virtue of that, this is one of the main used methods to determine phytohormones (WANG *et al.*, 2022).

2.1.4   UV-Vis Spectrophotometry

Ultraviolet absorption is used in several fields for different applications, since it is a reliable, simple, low cost and sensitive technique. As it does not need a large concentration of the compounds for the determination, it is possible to use a small amount of sample for the analyses. Since it is a versatile method, this technique is used over the years in some fields like environmental, pharmaceutical, agriculture and clinical. The wavelength range (nm) determines the spectral region of the absorbing substance is found, the energy range and the types of excitation necessary (PASSOS; SARAIVA, 2019).

2.2   Methods for Auxins Quantification

At present, there are many studies on different methods for the determination and quantification of auxins, but there are few studies using high-performance liquid chromatography (HPLC) and electrochemical determination combined. Since the determination of the concentration of these phytohormones is extremely difficult, reliable, accurate, simple, sensitivity, rapid, and quantitative analytical methods are required for their quantification. The most used methods are high-performance liquid chromatography (HPLC), previously described, and gas chromatography (GC). However, it is possible to use other methods like electrochemical analyses, immunoassay, spectrophotometric, thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), and micellar electrokinetic chromatography.

According to Wang *et al.* (2022), for the determination of phytohormones in microalgae, it is possible to use various methods, such as immunoassay determination and gas chromatography (GC). The immunoassay determination is used less frequently in this case, since it has some huge disadvantages such as poor repeatability, high interference, and unstable operation. This method provides the quantification and

detection of the analytes, based on using the link between antigens and antibodies, this last one is used as analytical reagents for the analysis. The GC is one of the most commonly used methods for this purpose due to its high sensitivity to the auxins. Besides, it has good separation, accuracy, and selectivity performance. However, it is an expensive method, whereas the equipment and their maintenance have a high cost. In addition, GC generally requires an elevated pre-treatment workload to prepare the sample for the derivatization process to carry out GC analysis, since it is necessary for the analyte to have low gasification temperature and polarity.

According to Goswami *et al.* (2015), for the determination of phytohormones, including IAA and IBA, in rhizobacteria, a method using high-performance thin-layer chromatography (HPTLC) was accomplished in the sample. For this type of sample, it is common to use spectrophotometry to determine indole compounds (mainly IAA). Although an easy method, it is inaccurate since it reacts with all the indole compounds present in the sample, quantifying the total indole rather than detecting them individually. On the other hand, it is possible to use thin-layer chromatography (TLC) to separate compounds in a mixture. It is a quick and simple method of planar chromatography; however, it only provides qualitative detection of indoles present in the sample. Thus, an improved technique of TLC using HPLC was proposed, HPTLC. It is a simple, quick and robust method with high separation efficiency, since it provides IAA and IBA concomitant determination in the sample without other indoles interference. Then, it is a helpful method to researches using samples produced by bacteria.

According to Bulícková *et al.* (2013), for the determination of IAA in an aqueous solution, an electroanalytical method was used with a glassy-carbon electrode. Combining a purification step (extraction of plant tissue and their pigments separation) with differential pulse voltammetry, using a three-electrode cell. It is a fast, selective and inexpensive technique. The auxin was quantified under the optimized conditions and the value of quantification limit (LOQ) of IAA reached the value found in the literature. In addition, the linearity of the concentration curve also verified the method to determine this auxin.

According to Han *et al.* (2011), for determination of phytohormones, including IAA and IBA, in grapes, a method using high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) was accomplished in the sample extracting and purifying them to their quantification. The IAA and IBA limits of quantification (LOQ) and concentration linearity under the optimized conditions verified the applicability of this method to a fast determination of the auxins in multiresidue analyses.

According to Sun *et al.* (2013), a method using micellar electrokinetic chromatography was accomplished for the determination of phytohormones, including IAA and IBA, in mungbean sprouts. Under the optimized conditions, the auxins were quantified and the results of IAA and IBA limit of quantification (LOQ),

concentration linearity, precision and accuracy confirmed the applicability of this convenient, rapid and low-cost method to determine these auxins.

As can be observed, the methods before mentioned were used to quantify and/or determinate IAA and IBA in indole's samples. Even so, in the literature some of them reached good results for the auxin quantification, there is a gap when it comes to the joining of HPLC with the amperometric detector. At the time of this work, no paper using this specific combination was found. Therefore, the following work has the purpose of filling this gap.

3   Materials and Methods

This research was accomplished during a 3 months volunteer internship exchange in Prague, Czech Republic. The research was carried out from January to April 2018 by the author of this work at the UNESCO Laboratory of Environmental Electrochemistry located at Charles University under the supervision of RNDr. Hana Dejmková, Ph.D. This study had the main aim of developing a new detection method using amperometric detector equipment with carbon felt electrode combined with HPLC for simultaneous analysis and determination of IAA and IBA.

3.1   Chemical and Samples

3.1.1   Stock Solutions

The indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were acquired from Sigma–Aldrich and used to prepare stock solutions of concentration 1.10^{-3} M (mol/L): 4.4 mg of IAA was dissolved in 25 mL of water, 5.1 mg of IBA was dissolved in 25 mL of mixture methanol: water (1:9, v/v). The solutions were kept in the dark at low temperatures (under 10 °C).

The stability of the prepared solutions was tested spectrophotometrically against blank solutions. Besides, the maximum absorbance was determined from the spectrometric measurements. For the injection in HPLC during the optimization, a solution with 1.10^{-4} M of IAA and IBA was prepared by diluting the stock solution with water.

3.1.2   Buffer Solution and Mobile Phase

The acidic part of the buffer is composed by mixing 2.45 mL of H_3PO_4 85% (Lach-Ner, Czech Republic) and 2.29 mL of CH_3COOH (Lach-Ner, Czech Republic) with water in a 1000 mL volumetric flask and basic part consists of 0.2 M NaOH (Fluka). Buffer is obtained by the mixing of these parts until the appropriate pH is reached. The mobile phase used on the HPLC system was prepared by combining 0.05 M phosphate-acetate buffer with methanol (gradient grade, Biosolve, Netherlands).

3.1.3   Rooting Preparation - Stimulax II

The rooting preparation Stimulax II was bought from a local gardening shop in Prague, Czech Republic. This sample is a liquid with declared composition of 0.06% IAA and 0.05% IBA. By the time it was purchased and used for the research, 13 months had passed since it was produced. However, its manufacturer describes a 3 years shelf life, thus it was not expired and should have presented the composition written on the label. For the sample preparation to inject in HPLC to quantify IAA and IBA presents, 0.15 mL of Stimulax II was diluted with 10 mL of H_2O in a volumetric flask.

3.2   Instruments

Spectrophotometric measurements were performed using spectrophotometer Specord 210 plus (AnalytikJena, Germany) with 1 mm quartz cuvettes.

The HPLC consisted of degasser, high-pressure pump Beta 10, injector valve with 20 mm^3 loop (all Ecom, Czech Republic), column LiChroCART 125-4, LiChrospher 100, RP-18 (5 µm) (Merck), electrochemical detector ADLC 2 (Laboratorni pristroje Praha, Czech Republic), and UV detector Sapphire 600 (Ecom, Czech Republic).

The electrochemical detector was set in the three-electrode arrangement. The Ag/AgCl (3 M KCl, ETP CZ R-008-05, Monokrystaly Turnov, Czech Republic) reference electrode and the platinum wire (ETP CZ P-002-05, Monokrystaly Turnov, Czech Republic) auxiliary electrode were used. The working electrode was made from carbon felt cylinder with diameter and thickness of 5 mm (Karbotechnik, Czech Republic). The felt was attached in the outlet hole together with the platinum electrical contact, the last two helped adjusted the correspondent screw cap, which was set with the screw with flat ferula. The scheme of this detector is represented in Figure 6 (DEJMKOVÁ *et al.,* 2017).

Figure 6 - Carbon felt detector's scheme. Inlet (1); Screw with flat ferula (2); Cap (3); Carbon felt (4); Outlet (5); Platinum electrical contact (6); Reference electrode (7); Auxiliary electrode (8); Mobile phase in an overflow vessel (9).
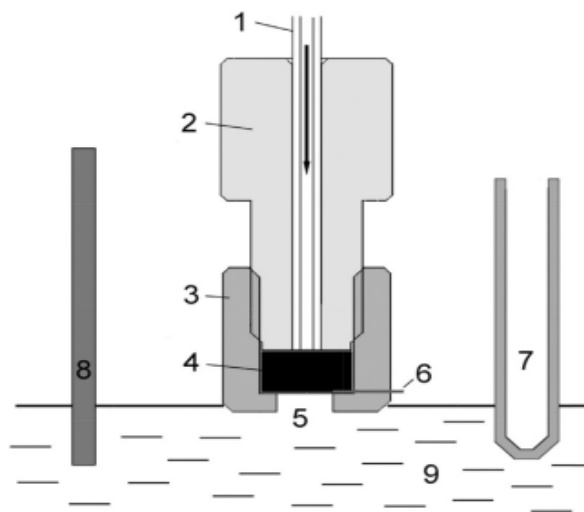
Source: (DEJMKOVÁ *et al.,* 2017).

3.3 Procedures

Statistical evaluation of the concentration dependences was made by the regression method. Determination limits (LOQ) for both compounds were calculated for the lowest concentration's solution through Equation (3.1). The variable "error" is the standard deviation.

$$LOQ = \frac{\text{"error"} \times 10}{slope}$$

Equation (3.1)

4   Discussion of Results

The methodology previously reported and the results obtained have already been published in an International Journal of Chemistry, "Monatshefte für Chemie - Chemical Monthly", in the article "Electrochemical determination of indole-3-acetic acid and indole-3-butyric acid using HPLC with carbon felt detector" (DEJMKOVA; DANIEL, 2019). This undergraduate dissertation will debate deeper the results already achieved.

Stock and buffer solutions were prepared and they were used to accomplish a series of experiments to determine the best conditions and operation mode to set in the equipment to optimize the results. The purpose was to corroborate the applicability of this method to determine the auxins IAA and IBA in the rooting preparation by comparing the concentration dependence with the reference method (HPLC-UV). Therefore, if it proves to be efficient and accurate, it will be a cheap and alternative technique to their quantification, which might help future researchers who work with gardening products and plant samples.

4.1   Method Optimization

To determine the best parameters to set in the equipment during the analyses to optimize the results, it was necessary to accomplish several analyses to find the maximum absorbance, the best percentage of methanol, pH and potential.

4.1.1   Absorbance

First, UV-Vis spectrophotometry was used against the blanks solutions to determine the stability of the IAA and IBA stock solutions. Thereafter, the maximum absorbance was determined from the UV-VIS spectra of the 1.10^{-4} M mixed solution of IAA and IBA. From the graphic represented in Figure 7, UV spectrum, the wavelengths nearest 220 nm exhibited the highest peaks for the two compounds. The magnitude of these peaks confirms the maximum absorbance for this kind of sample. Therefore, this value was set in the HPLC for further experiments.

Figure 7 - UV-Vis spectrum of indole-3-acetic acid and indole-3-butyric acid.
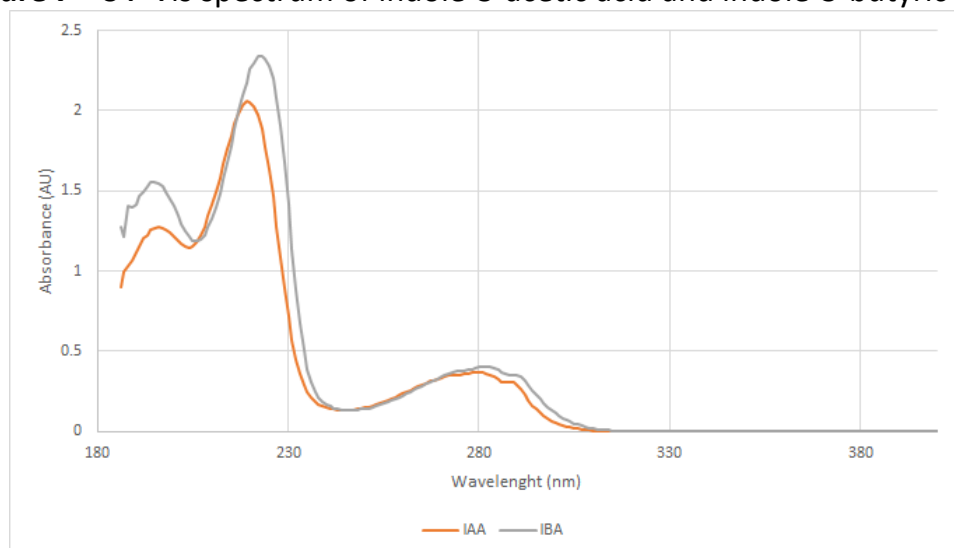
Source: Elaborated by the author.

4.1.2 Percentage of methanol

To set the optimum separation conditions, three different percentages of methanol in the mobile phase were tested. Solution of the mixed indole compounds of the concentration 1.10^{-4} M was injected using mobile phase containing 50, 60 and 70% of methanol in mixture with phosphate-acetate buffer.

From the chromatogram represented in Figure 8, 60% of methanol was the best percent to work with, since in this value the peaks were more separated than in 70% and the separation was faster than in 50%. Therefore, this value was set in the HPLC for further experiments.

**Figure 8 -** Chromatogram for 50, 60 and 70% of methanol in mixture with phosphate-acetate buffer. The first peaks of each percentage are for indole-3-acetic acid and the second for indole-3-butyric acid.
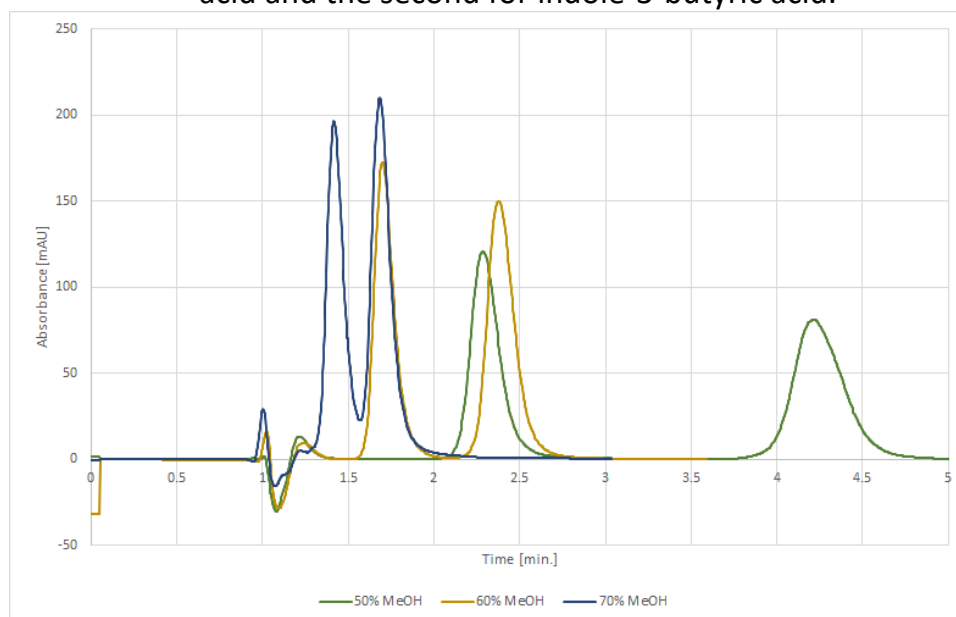
Source: Elaborated by the author.

4.1.3 pH and potential

The optimum potential of the working electrode and the pH of the mobile phase needs to be found. The measurements were accomplished in triplicate for 3 different buffer solution pH, namely 2.5 (represented in Figure 9), 4.0 (represented in Figure 10), and 6.0 (represented in Figure 11). Under each condition, the solution of the analytes of concentration 1.10^{-4} M was injected while at the same time the potential of the amperometric detector was changed. Under pH 6.0, it was not possible to separate the peaks of the compounds, so the solutions of each compound were injected separately.

Figure 9 - Voltammogram for buffer solution pH 2.5.
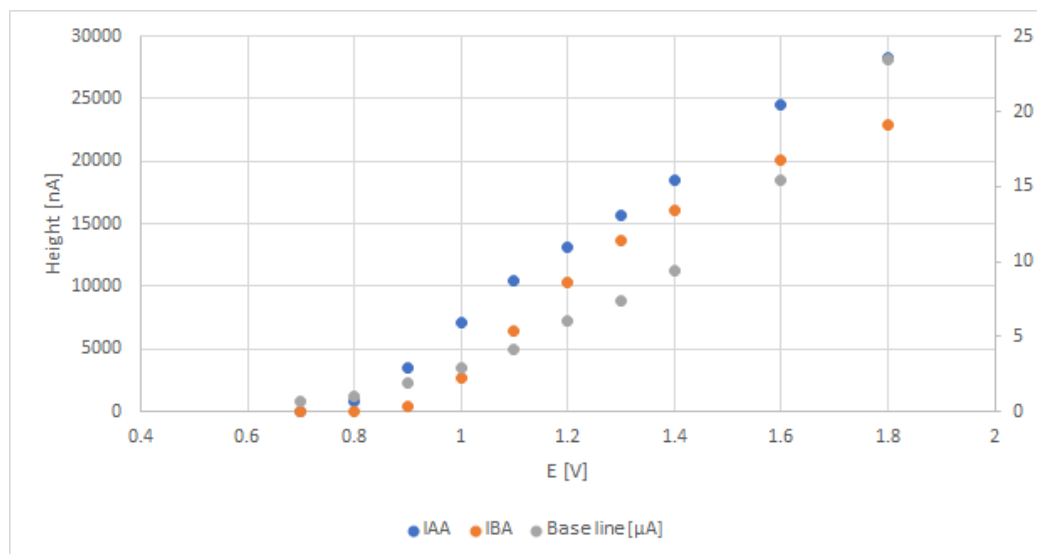
Source: Elaborated by the author.

Figure 10 - Voltammogram for buffer solution pH 4.0.
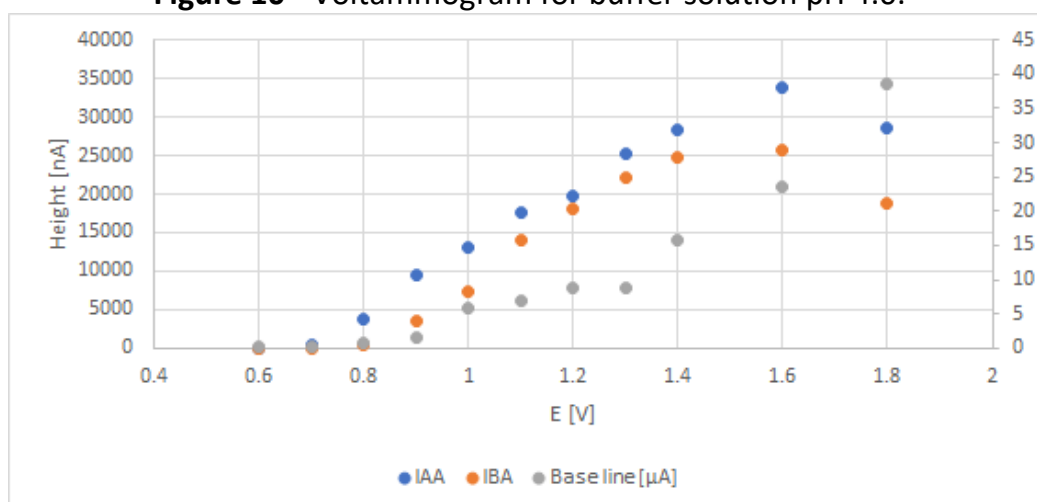
Source: Elaborated by the author.

Figure 11 - Voltammogram for buffer solution pH 6.0.
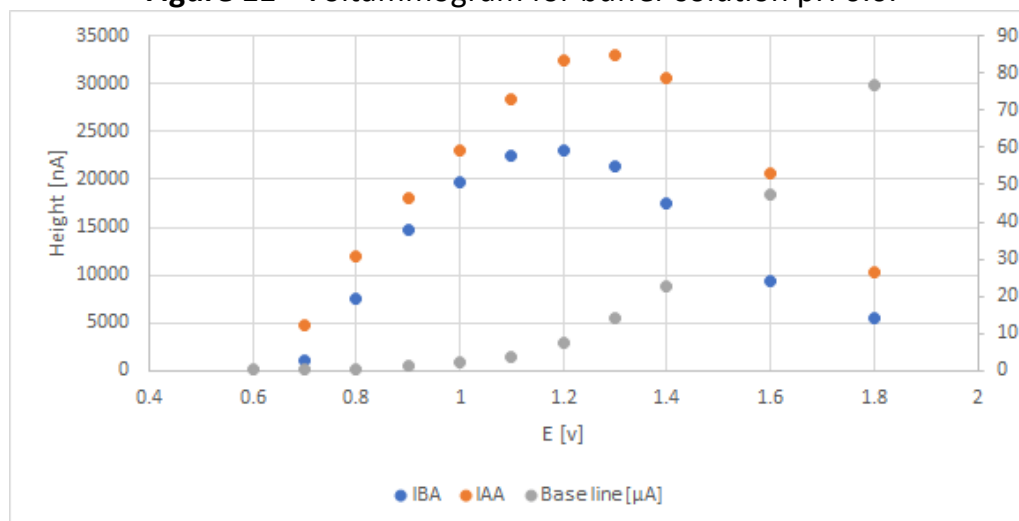
Source: Elaborated by the author.

In order to better explain and visualize the different pHs for each separate compounds and baseline, the three graphics represented in Figure 12 (A), (B) and (C) were assembled.

Figure 12 - Voltammograms for buffer solution pH 2.5, 4.0, and 6.0: (a) indole-3-acetic acid; (b) indole-3-butyric acid; (c) background.
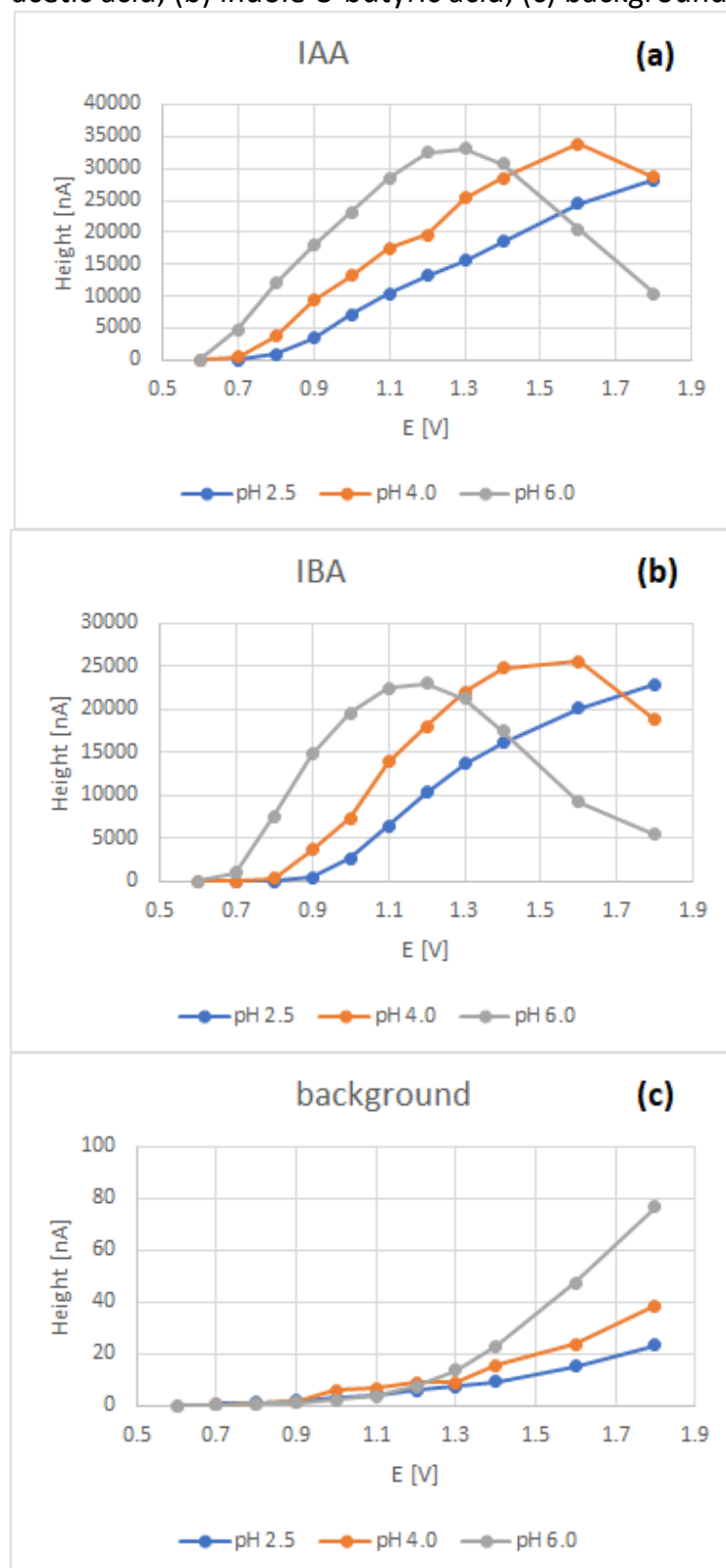
Source: Elaborated by the author.

Analyzing the graphics in Figure 12, it was possible to determine the best conditions to work. Observing the graphics for IAA and IBA, in the potential 1.4 V the curve is still growing, it has not reached its peak yet. The potential 1.6 V could be considered as the limit of the safe zone. Then, the most appropriate potential to work was between them, +1.5 V, since nearest it showed the biggest peak and lowest background current.

Due to the deproteinization, the phenomenon when the transfer of a proton forms a conjugate base. The retention time (t_R) had different behaviors for pH under (2.5 and 4.0) and above (6.0) the compound's pKa (4.8 for IAA and 4.7 for IBA).

As shown in Figure 13, even if the time were smaller to pH 6.0 (1.25 min and 1.46 min for IAA and IBA, respectively), this pH was not satisfactory because of the retention loss. In addition, it did not allow to work with the analytes' solution, only with them segregated.

For pH 2.5 and 4.0 the times were higher but still quite fast, 1.80 min and 2.47min for IAA and IBA, respectively. However, pH 2.5 was worse than 4.0, due to lower peaks height and the necessity to apply higher potential. Consequently, pH 4.0 was chosen for further experiments.

Figure 13 - Comparison of the time for the pH's 2.5, 4.0, and 6.0 in E_{DET}=+1.6 V. The first peaks of each percentage are for indole-3-acetic acid and the second for indole-3-butyric acid.
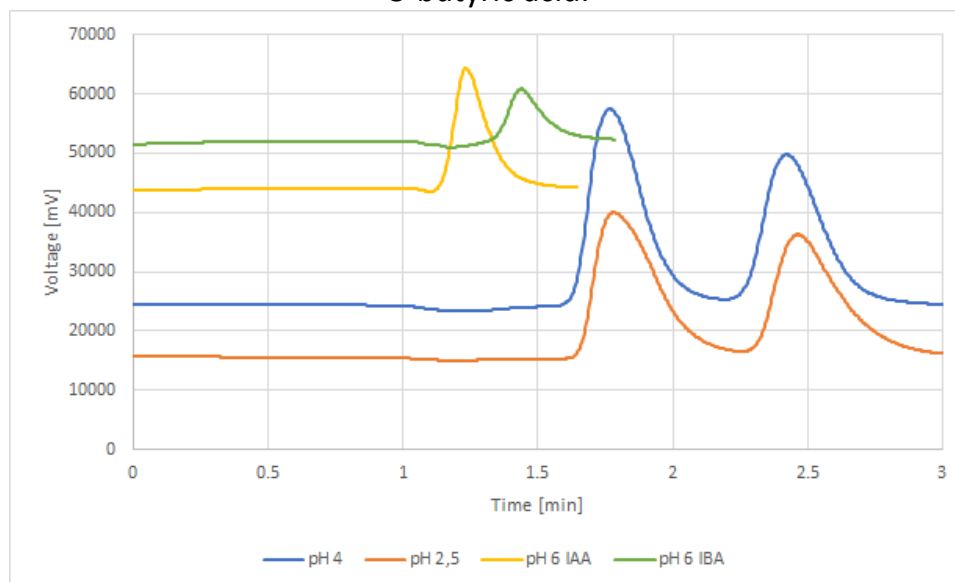
Source: Elaborated by the author.

4.2   Analyses to verify the methodology

To verify the efficient of the methodology, the best parameters obtained were set in the equipment to optimize the results. The absorbance was checked and tests of repeatability, concentration dependences and rooting preparation, Stimulax ll, were accomplished using the HPLC-ED and HPLC-UV, as reference method, for the analyses.

4.2.1   Confirming the Absorbance

With the purpose of confirming that the UV spectra did not change under pH 4.0, another test with UV-Vis spectrophotometry was performed. Two solutions were prepared from the stock solution for each one of the compounds with methanol and buffer solution pH 4.0.

The results of this analysis are shown in the graphic in Figure 14. After comparing this graphic with Figure 7, it was possible to confirm that the best wavelength to work continues to be 220 nm.

Figure 14 - UV-Vis spectrum of indole-3-acetic acid and indole-3-butyric acid with buffer solution pH 4.0.
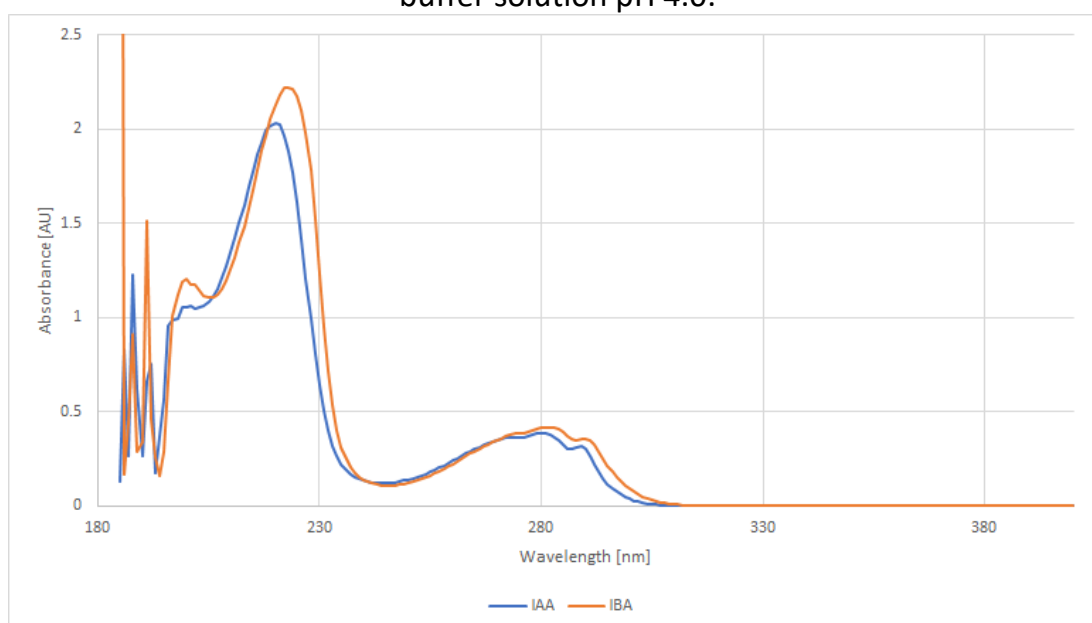
Source: Elaborated by the author.

4.2.2   Repeatability

After setting the equipment with 220 nm, 60% methanol, pH 4.0 and +1.5 V, the repeatability of the analysis was checked. The analyses were carried out by injecting 10 times the indole compounds mixture changing 3 times the carbon felt. These measurements had the objective of confirming the stability of the analyses.

From the graphics in Figure 15, it was concluded that the IAA had an unexpected behavior, since the current increased with the measures for the three felts. Meanwhile, the IBA had a random but expected behavior. Since the measures for each felt, the current kept similar between them.

Figure 15 - Current versus Measures for 3 different carbon felts: (a) indole-3-acetic acid; (b) indole-3-butyric acid.
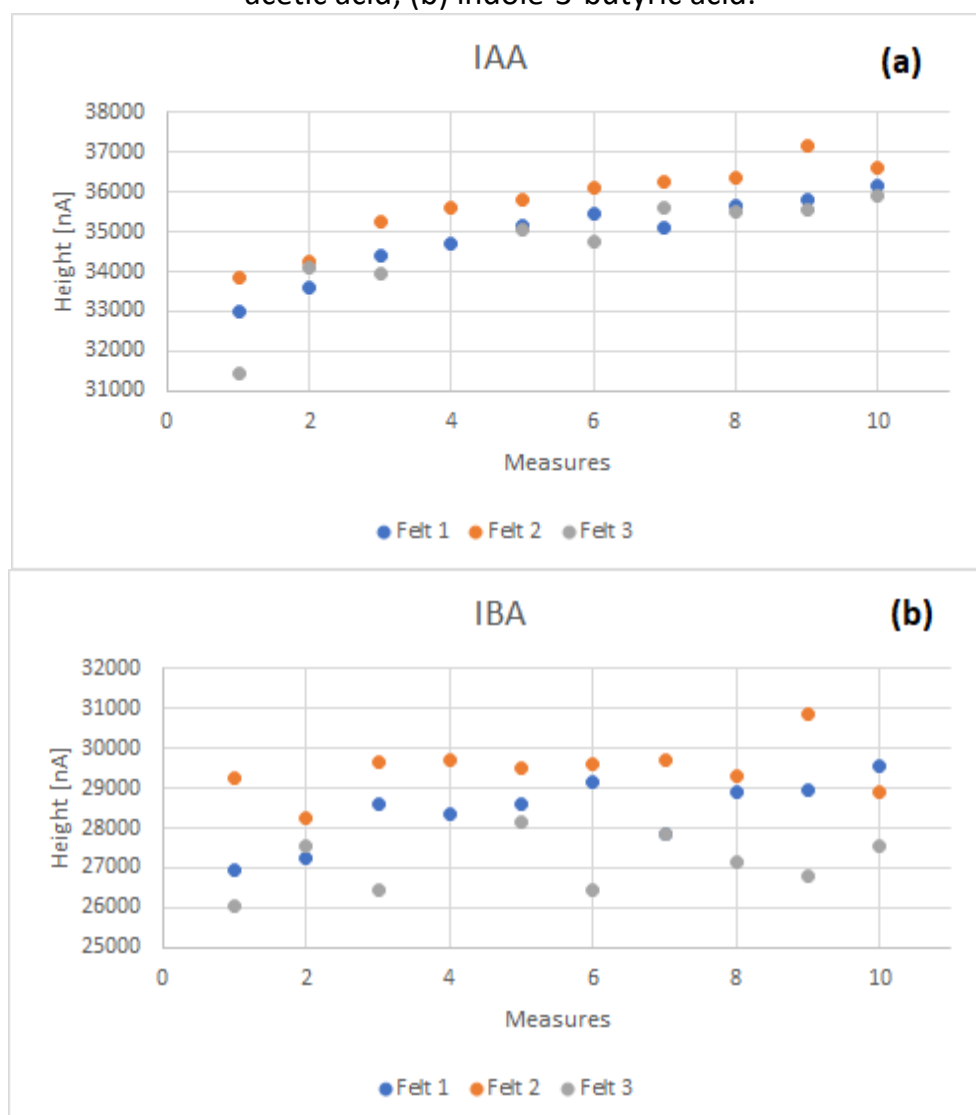
Source: Elaborated by the author.

Through Table 2, it was possible to visualize and confirm the stability of the analyses, since the values for the relative standard deviation of IAA and IBA were 3.1% and 2.5%, respectively.

Table 2 - Repeatability results.

Analyte	FELT 1	FELT 2	FELT 3	Relative Standard Deviation
IAA	2.7%	2.7%	3.8%	3.1%
IBA	2.8%	2.1%	2.5%	2.5%

Source: Elaborated by the author.

4.2.3 Concentration Dependences

The measurements were accomplished to verify the parameters of the concentration dependence through the observation of their linearity and detection and determination limits reached for IAA and IBA. They were executed in the concentration range from 4×10^{-7} to 1×10^{-4} M. The lowest concentration, which was possible to determine with HPLC and amperometric detection, was measured 10 times and its standard deviations were 0.040 and 0.052 µA for IAA and IBA, respectively.

Through the graphic from Figure 16, it was possible to confirm the linear behavior by the least squares linear regression method, since the correlation coefficients (R^2) were almost 1.0. Table 3 shows the correlation coefficient, slope, intercept, standard deviation and LOQ, calculated from Equation (3.1) using the standard deviation of the lowest concentration (4×10^{-7} M). The values reached for IAA and IBA's LOQ were approximately 1.1 and 1.8 µmol dm^{-3}, respectively. They were enough for their determination in plant samples. The intercepts are insignificant.

Figure 16 - Dependence of the current's peak on increasing indole-3-acetic acid and indole-3-butyric acid concentrations, analyzed with HPLC-ED.
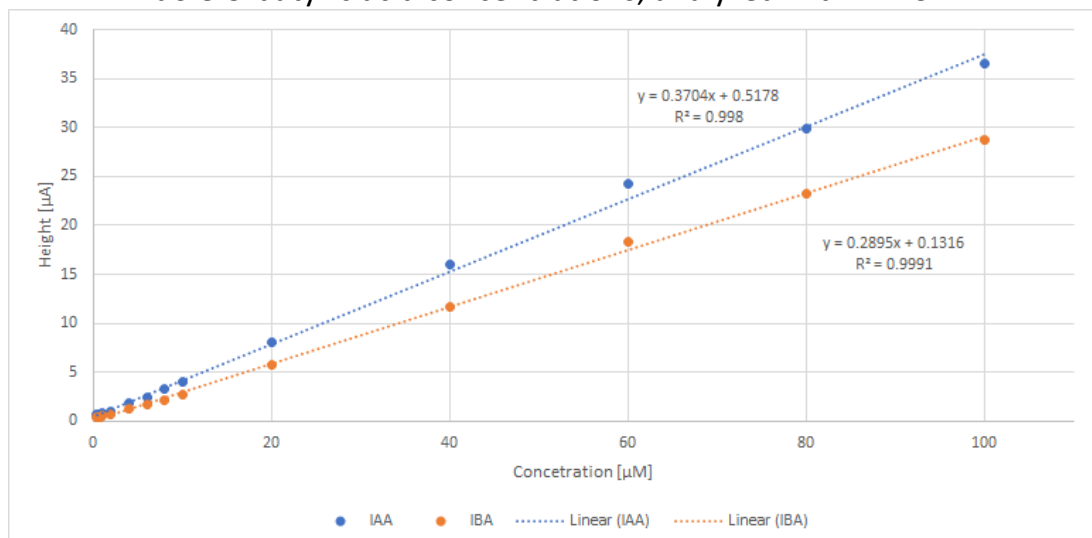
Source: Elaborated by the author.

Table 3 - Values for correlation coefficient, slope, intercept, standard deviation and LOQ of indole-3-acetic acid and indole-3-butyric acid for HPLC-ED measurements.

Analyte	Correlation Coefficient	Slope (A dm3 mol-1)	Intercept (µA)	standard deviation (µA)	LOQ (µmol dm-3)
IAA	0.9980	0.3704	0.5178	0.040	1.09
IBA	0.9991	0.2895	0.1316	0.052	1.79

Source: Elaborated by the author.

In the Figure 17, selected chromatograms obtained during the measurement of the concentration dependence are shown. The analyses proved to be quite fast, less than 3 minutes.

Figure 17 - Chromatograms of the solution of indole-3-acetic acid and indole-3-butyric acid mixture.

Source: Elaborated by the author.

4.2.4   Rooting Preparation - Stimulax II

For the analyses, the same previous conditions were kept, concentration of the analyte solution 1.10^{-4} M, wavelength of 220 nm, 60% of methanol, pH 4.0, and potential +1.5 V; the experiment was accomplished in triplicate. The main purpose of these measurements was to check the veracity of what the manufacturer declared on the label of this product. As well, it was possible to verify the efficiency of the determination method of IAA and IBA through HPLC-ED. HPLC-UV was used as the reference method; its concentration dependences are shown in Figure 18.

Figure 18 - Dependence of the current's peak on increasing indole-3-acetic acid and indole-3-butyric acid concentrations, analyzed with HPLC-UV.
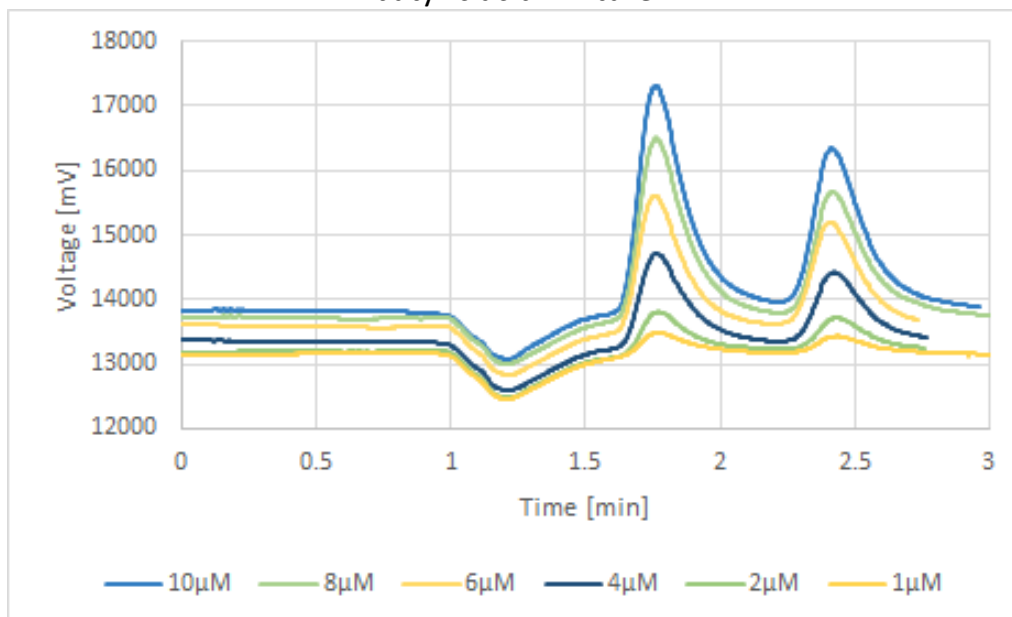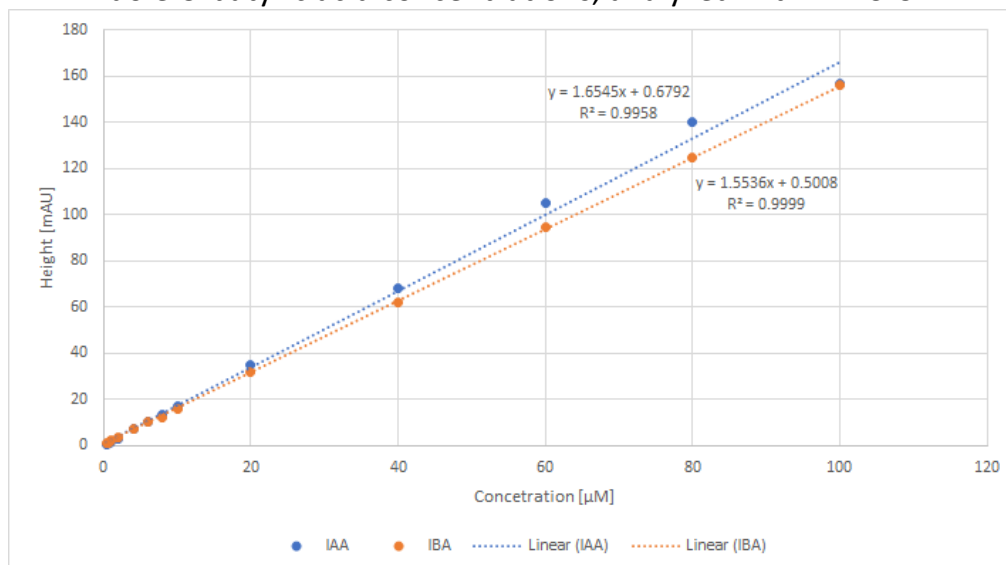

Source: Elaborated by the author.

Applying the calibration equations, Equation (4.1), it was possible to calculate the concentrations. The results for the two analytes in both methods are shown in Table 4.

$$I = a * c + b$$

Equation (4.1)

Wherein "a" and "b" are the calibration equations' slope [A L mol^{-1}] and intercept [µA], respectively; "I" is the average of the height in µA; "c" is the compounds' molar concentration [M].

Table 4 - Values for average height and concentration of indole-3-acetic acid and indole-3-butyric acid for HPLC-ED and HPLC-UV measurements.

	Analyte	Average of Height [µA]	c [M]
HPLC–ED	IAA	4.06	9.56E-06
	IBA	9.63	3.28E-05

	Analyte	Average of Height [µA]	c [M]
HPLC–UV	IAA	16.59	9.62E-06
	IBA	50.15	3.20E-05

Source: Elaborated by the author.

Through the obtained concentration values, the percentages of each compound in the sample were found. Table 5 allowed to compare the results of these two methods with the manufacturer's declared, 0.05% and 0.06% for IAA and IBA, respectively. The HPLC-ED and HPLC-UV methods were accurate since their results are similar. However, the agreement between the measures of these two determination methods and the declared by the manufacturer was distinct, reaching around 20% for IAA and 70% for IBA of the announced values.

Table 5 - Comparison of HPLC-ED, HPLC-UV and manufacturer's declared content of indole-3-acetic acid and indole-3-butyric acid in the rooting preparation sample, Stimulax ll (variability expressed as standard deviation).

Analyte	HPLC–ED deter-mined content/%	HPLC–UV deter-mined content/%	Declared content/%
IAA	0.0112 ± 0.0001	0.0112 ± 0.0002	0.05
IBA	0.0445 ± 0.0002	0.0433 ± 0.0012	0.06

Source: (DEJMKOVA; DANIEL, 2019)**.**

5   Conclusion and Future Works

The present work had the objective of confirming the suitability for HPLC method using carbon felt as electrochemical detection (HPLC-ED) in indole compounds, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Firstly, a deep literature search was conducted with aim of comparing the different existent quantification methods for the auxin determination and/or quantification and proving that there is a missing gap when it comes to this specific combination.

Performing several tests, the optimized parameters were acquired. By UV-vis spectrophotometry, a wavelength of 220 nm was obtained as the maximum absorbance. A mix solution combining IAA and IBA with a concentration of 1.10^{-4} M was used to inject in HPLC along the experiment.

The mobile phase was kept isocratic elution. The best percentage for working was 60% of methanol, whereas it was faster than in 50% and had more separated peaks than in 70%.

The values for pH and potential were obtained using HPLC-ED. The tests were accomplished by changing the potential along the three different pHs. The most suitable pH to use was 4.0, since it had higher retention of the auxins. For the potential, +1.5 V exhibited the lowest background current and biggest peak.

HPLC using carbon felt as a detector confirmed the stability of the method, since it provided high repeatability with a felt exchange, showing a relative standard deviation of 3.1% and 2.5% for IAA and IBA, respectively.

In addition, the linear behavior was proven by the least-squares linear regression method since the correlation coefficients (R^2) were around 1.0. The determination limits (LOQ) reached 1.1 µmol dm^{-3} for IAA and 1.8 µmol dm^{-3} for IBA. Thus, these values were enough to determine the auxins in plant samples and gardening products, as in the case of this research.

The results indicated that the method of HPLC-ED reached approximately the same values for the indole compounds concentration in the sample as with HPLC-UV, which was used as the reference method. It confirmed the efficiency of this determination method for the considered compounds.

Using calibration dependence, the compound concentration in the rooting preparation, Stimulax II, was achieved. However, even the values for the two HPLC methods were in agreement, around 0.01% for IAA and 0.04% for IBA. They did not reach the declared by the manufacturer in the label, which was 0.05% and 0.06% for IAA and IBA, respectively. Through these analyses, it was possible to check that IAA is more unstable than IBA, showing lower content in the sample.

It was concluded that even if the new detection method (HPLC-ED) for simultaneous analysis and determination of both compounds was accurate, the gardening product, Stimulax II, had distinct values. The results for the tests only

reached around 20% and 70% of the declared values for IAA and IBA, respectively. The fact that these values were not obtained could be associated with the instability of the auxins and/or their degradation in the product. A suggestion is the manufacturer review the proposed shelf life.

For future works, it is suggested to use the same techniques, HPLC with carbon felt as a detector, since it has proven to be a good alternative method, it is cheap and quite fast (separation of the analytes in less than 3 minutes). As well, HPLC with UV spectrometric detection can be used as the reference method. Therefore, others plant samples, as gardening products, might be analyzed and checked to determine if the indole compounds concentrations are divergent as they are in the case of Stimulax II.

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