IOSUD – "DUNĂREA DE JOS" UNIVERSITY OF GALATI
Doctoral School of Fundamental Sciences and Engineering

PhD THESIS

ABSTRACT

DEVELOPMENT OF NEW VOLTAMPEROMETRIC SENSORS AND BIOSENSORS FOR THE DETERMINATION OF HYDROXYCINNAMIC ACIDS

PhD student,

Alexandra Virginia MEREŞESCU (BOUNEGRU)

Scientific coordinator,

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Mereşescu (Bounegru) Alexandra Virginia

CONTENTS

(The numbering of the pages corresponds to the doctoral thesis)

INTRODUCTION

Biologically active substances have the property of interacting through physical, physio-chemical, or chemical mechanisms with the molecules, atoms or ions of which living matter is composed, triggering a succession of reactions in the body.

Hydroxycinnamic acids are organic compounds derived from the cinnamic acid, with an aromatic nucleus, at least one phenolic group, an aliphatic double bond, and a carboxyl group.

These compounds have multiple physiological functions, such as antioxidant, anti-inflammatory, anti-microbial and anti-melanogenic activity. These properties are the basis for the increasing use of hydroxycinnamic acids and their derivatives in pharmaceutical formulations, food supplements and cosmetics. Pharmacological potential of these compounds can be explained by the presence of one or more hydroxyl groups in their chemical structure, thus giving them the ability of removing free radicals [1].

In this paper, we have chosen to study some of the hydroxycinnamic compounds, namely, caffeic acid, ferulic acid and p-coumaric acid.

Caffeic acid is one of the most common phenolic acids that are found in fruits, vegetables, mushrooms, and herbs. It is biosynthesized by hydroxylation of p-coumaric acid. Caffeic acid is often found in food supplements in various proportions, having antioxidant, antitumor, anti-inflammatory, antimicrobial, and antidiabetic actions [2].

Ferulic acid is a hydroxycinnamic acid, present in many fruits, vegetables, rice, and some herbs. This phenolic compound has an important role in the human body and is absorbed in the small intestine as a major metabolite of chlorogenic acid. Ferulic acid is often used in various types of cosmetics, such as sunscreens, cleansers, moisturizers, or anti-wrinkle creams, as it can stimulate intracellular defence systems. Ferulic acid may also be useful in topical products with pharmacological role. Depending on the quantity, it can be an adjunct in the treatment of atopic dermatitis. Therefore, the determination of ferulic acid concentration is important in the nutraceutical and cosmetic industry [3].

P-coumaric acid is produced mainly as a phenolic acid from tyrosine and phenylalanine. It is a major precursor in the synthesis of other phenolic acids, such as caffeic, chlorogenic, rosmarinic and ferulic acid. It is widely distributed in fruits, vegetables, grains, and mushrooms [4]. Studies on p-coumaric acid have shown multiple health benefits due to its antioxidant, antimicrobial, antitumor, anti-inflammatory, antiplatelet and healing properties [5,6].

Food supplements, phyto-homeopathic formulations, nutraceutical products, medical devices and much of cosmetics and care do not receive very detailed booklets on concentrations of active ingredients. In certain situations, the quantification of these compounds and the ratio between them may be relevant to optimize treatments or maintain proper health.

In recent years, electrochemical analyses have been proved to be promising in various areas such as quality control in the food, pharmaceutical or cosmetics industries, due to their high sensitivity, low detection limit and relatively low costs. In applying this method, the analyte concentration is determined by measuring the analytical signal (electric potential or current).

One of the most widely used electrochemical techniques is cyclic voltammetry. It consists of applying a potential and record the system response time or current due to electrochemical processes that occur in the system.

In this paper, cyclic voltammetry was most often used to obtain information on the mechanism of the electrochemical reaction, the identification of the species in solution and the determination of the diffusion coefficients of the electroactive species. Square wave voltammetry and chronoamperometry have also been used in some studies.

Thesis entitled: DEVELOPMENT OF NEW VOLTAMMETRIC SENSORS AND BIOSENSORS FOR DETERMINATION OF HYDROXYCINAMIC ACIDS aims to highlight the effectiveness of voltammetric methods and detection instruments (sensors or biosensors) for the qualitative and quantitative analysis of food supplements, phytopreparations or cosmetics.

The paper is structured in two parts and eight chapters. The theoretical part presents current reviews on hydroxycinnamic acids, the development of screen-printed sensors, ways of

functionalization for increasing analytical performance, nanomaterials used to modify screen-printed electrodes, features and advantages of carbon-based nanomaterials, biometric techniques, and notions about immobilization of enzymes laccase and tyrosinase, both used for the experimental studies in this paper.

The originality of the thesis consists in experimental studies on evaluating the electrochemical behaviour of new sensors and biosensors in different types of electroactive solutions, optimizing parameters for solutions with different pH values, modifying sensors to increase sensitivity and selectivity, determining parameters such as active surface area of the electrode, the diffusion coefficient, the concentration of active species on the surface of the electrode, the limits of detection and quantification and analytical determination of the hydroxy acids from different products.

Immobilization of enzymes (laccase and tyrosinase) on previously modified surfaces with various nanomaterials or organic compounds has been proved to be successful in increasing selectivity in the detection of hydroxycinnamic acids.

The feasibility of voltammetric methods and the performance of the enzymatic sensors and biosensors developed were validated by similar results obtained with spectrometric and chromatographic methods.

The results showed that the test products benefit from significant amounts of antioxidant substances, predominantly the hydroxy acids, according to the manufacturers specifications.

The tools developed and optimized in this study could be used for routine testing of such analytes in products with different presentations, bringing further innovation in current and future research.

Keywords: hydroxycinnamic acids, sensor, biosensor, voltammetry, enzyme, nanomaterials, carbon nanofibers.

PART I. STATE OF KNOWLEDGE
CHAPTER 1. General considerations on phenolic compounds

1.1. Prevalence and biological activity

Phenolic compounds are a main class of secondary metabolites in plants and are classified into simple phenols and polyphenols. These compounds are found combined with mono- and polysaccharides, bound to one or more phenolic groups, or may appear as derivatives such as methyl esters [1].

Classification and structural features

Hydroxycinnamic acids (HC) shows a basic skeleton, C3 -C6 (phenylpropanoid). The main HCs are p-coumaric acid, caffeic acid, and methylated forms from ferulic and synaptic acids. However, like other phenolic acids, HCs are almost always bound to small or large molecules. The most abundant derivative of hydroxycinnamic acid in plant foods is an ester of caffeic and quinic acids, named chlorogenic acid.

Hydroxycinnamic acids, their chemical structure, and their role in the human body

Hydroxycinnamic acids are phenolic compounds found in fruits, vegetables, and beverages (coffee, tea, wine, etc.). HC are polyphenolic compounds class with multiple biological activities. In vitro and in vivo tests have shown that HC exibit an important antioxidant, antimicrobial, anti-inflammatory, antidiabetic and even antineoplastic activity [7]. Moreover, HC are neuro- and cardioprotective and play a role in the prevention of osteoporosis.

The class of hydroxycinnamic acids includes coumaric acids (CoA), caffeic acid (CA), ferulic acid (FA), cinnamic acid (CnA), synaptic acid (SA), but also their derived compounds. CA is present in plants, mainly in the form of an ester of quinic acid, which is called chlorogenic acid (ChA). Rozmarinic acid (RA) is an ester of CA and 3,4-dihydroxyphenylacetic acid.

1.1.1. Caffeic acid, biosynthesis, therapeutic actions, methods of determination

Caffeic acid is found in the form of a monomer, as ester of organic acids, amides and glycosides [8] or in more complex forms, such as dimers, trimers and flavonoid derivatives. It can also be bound to proteins and other polymers in the cell wall of plants [9].

Studies made in vitro and in vivo had proved that CA and its derivatives have numerous beneficial physiological effects, such as antibacterial [10–12], antiviral [13–16], antioxidant [17–20], anti-inflammatory activity. [21–24], anti-atherosclerotic [25–27], immunostimulatory [28–30], antidiabetic [86], [87],cardioprotective [33,34], antiproliferative [35], hepatoprotective [36,37], anticancer [52],[87],[90–92] etc.

1.1.2. Ferulic acid, biosynthesis, therapeutic actions, methods of determination

FA and its precursors, p-coumaric acid (p-hydroxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid), are metabolized in lignin biosynthesis. Its antioxidant properties are due to the possibility of forming the delocalized radical and phenolic nucleus. Since many natural compounds bear one or more feruloyl groups in their skeleton that presents interesting bioactivities, it is important to compare both the structural formulas, and natural bioactivity of the ferrule compound already reported in the literature [42].

1.1.3. P-coumaric acid, biosynthesis, therapeutic actions, methods of determination

Coumaric acids are cinnamic acid derivatives that are monohydroxylated to the phenyl group. P-coumaric acid (PCA) is the most abundant isomer [5,43]. This compound has many important applications in the nutraceutical, pharmaceutical and chemical industries. P-coumaric acid has antioxidant, antibacterial and anti-inflammatory properties and is used to produce flavours and fragrances for chemical or food industry. PCA is also a raw material for the preparation of biodegradable thermoplastic materials [44]. Recently, it has been discovered that

PCA also has an antiproliferative [45], anxiolytic [46], neuroprotective effect [47] and a nephroprotective role [45] and in addition can inhibit melanogenesis [48].

CHAPTER 2. Voltammetric techniques used in phenolic compounds analysis

Electrochemical methods are robust and sensitive methods used for both qualitative and quantitative analysis for a wide range of analytes with different concentrations. Electrochemical methods are used not only for their sensitivity to smaller amounts of analyte, but also for the separation of ionic species [49].

2.1. Cyclic voltammetry (CV)

Cyclic voltammetry has become a very popular technique for electrochemical characterization of new systems and proved to be very useful in obtaining information about complex electrode reactions. CV is used to study the behaviour at the interface of electroactive species. The CV can obtain information on the mechanism of the electrochemical reaction, the identification of the present species in the solution or the determination of the electroactive species diffusion coefficients. In the CV, the potential of an electrochemical system is changed back and forth between the two limits of potential and the response of the current is measured in relation to the potential. Voltammograms are graphical representations of the working electrode current as a function of the counter-electrode current [50].

CHAPTER 3. Nanomaterials for producing electrochemical sensors

3.1. Use of carbon nanomaterials to produce sensors

Electrochemical carbon-based sensors are often used due to their high sensitivity, low cost, good stability and biocompatibility [51,52]. The most common carbon-based electrochemical sensors include glassy carbon electrodes, graphite, carbon nanoparticles, carbon fibres, carbon microspheres, etc. [53–57]. Carbon nanomaterials such as graphene, graphene oxide, fullerene, carbon nanocons, quantum carbon nanoparticles, carbon nanofibers, carbon nanotubes offer advantages such as high surface-to-volume ratio and large specific surface area, important features for detection of analytes [58]. Typically, the incorporation of carbon nanomaterials as part of the sensitive layer of an electrochemical sensor is done by deposition on a solid support. This process can be performed by various techniques, such as: solvent dispersion, carbon nanomaterial paste, screen printing.

3.2. Screen-printed electrodes

In recent years, has been of particular interest the use of screen-printed electrodes (SPEs) as electrochemical detectors or as translators for the manufacture of electrochemical sensors due to their characteristics and advantages over other analytical instruments, thus allowing their use in a variety of electrochemical applications in different fields. Among the advantages and characteristics of screen-printed electrodes are the following: low cost, disposable, small size, portability, design flexibility, easy integration into electronic circuits, the possibility of pre-treatment and modification.

Carbon-based nanomaterials offer the possibility of combining with other nanomaterials to manufacture composites that synergistically increase their properties. Such composites can increase the sensitivity and selectivity of the modified surfaces [59].

3.2.1. Graphene modified electrodes

Graphene (GPH) is obtained by various methods, such as mechanical delamination of the graphite, graphite treating with oxidative agents, chemical vapor deposition, etc. [60–62]. GPH is an extensive 2D carbon network, with a honeycomb-like hexagonal structure, with high sensitivity, high selectivity, good stability, low action potential and excellent electrocatalytic

activity [63]. It has many interesting properties, such as very large specific surface area, optimal conductivity and transparency, excellent mechanical strength and flexibility, good thermal and electrical conductivity and excellent electronic properties[64,65].

3.2.2. Fullerene modified electrodes

Fullerene or "C60" is a class of carbon compounds, which generally have structures either with spherical shapes of the geodesic dome type (C60, C40) or with cylindrical shapes of "cage type" (nanotubes) [59]. Fullerene-based electrochemical sensors are used primarily for their catalytic reproducible responses and high chemical stability. The electrochemical behaviour of fullerenes can be improved by changes in its structure. The sensitivity can be further improved by depositing various metal nanoparticles on the activated fullerene. The hydrophilic character of the fullerenes may be induced by functionalization with polar groups [66].

3.2.3. Carbon nanocons modified electrodes

Carbon nanocons (CNHs) are conical nanostructures that are usually 30-50 nm long and 2-5 nm in diameter. Due to the closed structure, they can be considered similar to fullerene. CNHs have certain advantages over CNT. They are produced in high yield at room temperature and without the toxic metal contaminants, which make them safe for the environment [67]. CNHs oxidation produces the oxygen-containing extended functionalization, which facilitates adsorption or immobilisation of the electrocatalysts and numerous enzymes.

3.2.4. Carbon nanotube modified electrodes

Carbon nanotubes (CNTs) are one-dimensional cylindrical tubes of sp^2 hybridized carbon atoms. Their diameter is about tens of nanometres and their length may be up to several centimetres [68,69]. CNTs are classified according to the number of laminated graphene layers, so that we can have single-layer carbon nanotubes (SWCNTs), double-layer carbon nanotubes (DWCNTs) and multi-layered carbon nanotubes (MWCNTs). CNTs have some amazing electrical, mechanical, thermal and adsorption properties, which makes them optimal for the manufacture of electrochemical sensors [70]. CNTs substantially improve electron transfer and can be adequately functionalized with certain biomolecules for the manufacture of a biosensor.

3.2.5. Quantum nanoparticles modified electrodes

Quantum carbon nanoparticles (CD) and quantum graphene nanoparticles (GQD) have recently been presented as superior materials with multiple properties, which include excellent photostability, small size, biocompatibility, adjustable photoluminescence, exceptional multi-photon excitation capacity, electrochemiluminescence, easy functionalization with biomolecules and chemical inertia [71–74]. Due to their small size and biocompatibility, they can also serve as effective carriers for the administration of drugs. Moreover, due to their unique catalytic and physico-chemical properties, these materials can be used in various biomedical applications [75]. Quantum graphene nanoparticles (GQD) can be used as a kind of quantum carbon nanoparticles, which usually possess better crystallinity [76].

3.2.6. Carbon nanofibers modified electrodes

Nanofibers are characterized by the presence of many edges, which provide a surface adapted and available for interaction with gases, liquids or solids [77]. In recent years, nanofibers have become increasingly important due to the many advantages: high reaction speed and sensitivity, [78] the possibility of being synthesized from a wide variety of materials, optimal barrier to the diffusion of materials due to porosity and nanofiber interconnectivity [79], high activity of biological elements immobilized on the surface of nanofibers [80] and excellent structural mechanical properties [81,82].

CHAPTER 4. Enzymatic biosensors for the determination of hydroxycinnamic acids

4.1. General notions of enzymatic biosensors - types and classifications

Electrochemical biosensors are analytical devices similar to electrochemical sensors that incorporate biological molecules for quick and accurate detection of target species [83,84]. More specifically, biosensors are chemical sensors used for recognition properties of biomolecules from the electrode surface. They are used for the determination of biological molecules, pathogens or tumoral markers, very necessary in the medical field [85].

4.2. Immobilization strategies for development of enzymatic sensors

Choosing an appropriate immobilization technique is essential for the manufacture of biosensors [86]. One of the simplest methods of immobilizing the enzyme is by physical adsorption. Enzymes can also be immobilized by incorporation into three-dimensional matrices, such as an electropolymerized film [87,88], silica gel [89] or carbon paste [90]. Another immobilization technique very commonly used is crosslinking, but this involves the use glutaraldehyde [3,91]. Glutaraldehyde is a toxic reagent and may decrease the activity of the enzyme. Another conventional method of immobilization is covalent binding of enzymes. That is accomplished by initially activating the support using a coupling agent, followed by binding the enzyme to the activated surface. Enzymes can also be immobilized by affinity bonds between a functional support group and a specific molecule (e.g., biotin, carbohydrates, histidine) [92] naturally present or genetically modified, in an enzymatic sequence that does not affecting their activity [93].

4.3. Enzymatic biosensors for the detection of hydroxycinnamic acids

HC determination is made often using tyrosinase and laccase enzymes for the modification of the electrodes, due to their ability to oxidize these compounds [94]. The oxidation mechanisms are different and as a result either quinones are produced, from simple oxidation, or compounds that contain several oxygen atoms. The catalytic role of enzymes allows the amplification of electrochemical signals obtained by reducing the oxidation products of phenolic analytes

4.3.1. Tyrosinase-based enzymatic biosensors

Tyrosinase (Ty) is a metalloenzyme, which possesses two copper ions at active site, which are coordinated by three histidine residues in the enzymatic polypeptide chain [95]. Due to its ability to react with phenols, is useful for various applications in food, biomedical and pharmaceutical industries. Ty-based electrochemical biosensors can be considered "*classics*" because Ty is suitable for evaluating the electrochemical properties of materials, with the help of which new nanostructured electrodes are developed [96–98].

4.3.2. Laccase-based enzymatic biosensors

Laccase has been used in biosensors based on metal nanoparticles, carbon nanomaterials, polymers and biopolymers (chitosan) or various membranes such as Nafion. Of particular importance is the synergistic combination of laccase and nanomaterials that increase the performance of electrochemical biosensors [99]. To improve the stability of biosensors, an appropriate matrix of nanomaterials / metal nanoparticles and bovine serum albumin / β-cyclodextrin / bacterial cellulose can be achieved [100–102]. These biosensors have been used successfully for routine determinations in pharmaceutical samples [100] as well as determination of hydroquinone in water samples [101,103].

PART II. PERSONAL CONTRIBUTIONS

OBJECTIVES

The general objective of the paper was to develop and characterize new sensors and biosensors based on carbon nanomaterials, mediators of electron transfer and enzymes to determine hydroxycinnamic acids from nutraceuticals and cosmetics with different presentation forms, using voltammetric methods for detection.

Specific objectives:

• Characterization using cyclic voltammetry of screen-printed electrodes based on carbon nanomaterials in different electrolytes

• Application of the drop-and-dry technique, followed by crosslinking for the preparation of enzymatic biosensors, using the screen-printed electrodes based on carbon nanomaterials as support

• Optimization of the working parameters in model solutions

• Study of the electrochemical behaviour in electroactive solutions of sensors and biosensors

• Optimization of pH and other experimental parameters characteristic of the voltammetric technique to obtain a stable and reproducible electrochemical response

• Study of the reaction's kinetics at the working electrode

• Plotting the calibration curves and computation of detection and quantification limit for each sensor or biosensor used

• Study of the hydroxycinnamic acids determination by cyclic voltammetry or square wave voltammetry, from different pharmaceutical formulations or cosmetics

• Detection of interferences caused by other substances present in the composition of the analysed products

• Analysis of the repeatability and reproducibility of sensor and biosensor signals

• Validation of results with other analysis methods, such as Folin Ciocalteu, FTIR or UHPLC spectrophotometric method

• Determination of the antioxidant capacity of hydroxycinnamic acids in the Eye Blend product, by the DPPH method.

CHAPTER 5. Development of electrochemical sensors based on carbon nanomaterials for the determination of caffeic acid in food supplements

Introduction

The aim of this study was the electrochemical characterization of new carbon-based screen-printed electrodes modified with carbon nanofibers and multilayer carbon nanotubes, as well as the development and validation of an electroanalytical method for quantitative determination of caffeic acid in food supplements.

5.1. Results and discussions

5.1.1. Preliminary studies

In preliminary studies we evaluated the electrochemical properties of the three types of screen-printed carbon electrodes (SPCE), unmodified chemically modified with carbon nanofibers (CNF) and modified with multilayer carbon nanotubes (MWCNT). Electrolyte solutions were potassium chloride, a composed solution of potassium ferrocyanide and potassium chloride and a solution of catechol and potassium chloride.

There was obtained a stable signal when the potential of the sensor was situated in the range between -0.4 and 1.3 V in the case of caffeic acid. In the case of potassium ferrocyanide the optimum potential range was between -0.6 and +1.0 V.

5.1.1.1. Electrochemical properties of electrodes in inactive solutions

In the first stage was studied the voltammetric behaviour of the C-SPE, CNF / C-SPE and MWCNT / C-SPE electrodes in 0.1 M KCl solution (redox inactive substance) in the potential range between -0.6 and +1.3V. In the cyclic voltammograms no peaks were observed in the studied potential range. This demonstrates that the materials used to build the electrodes have a high purity, and that the electrodes do not show a contamination of the active surface, which could influence the electrochemical responses. However, background currents are noticeable lower when using CNF / C-SPE compared to the other electrodes used.

5.1.1.2. Electrochemical behaviour of C-SPE, CNF/C-SPE and MWCNT/C-SPE in electroactive solutions

In the next step, we studied the electrochemical behaviour of the electrodes immersed in a solution containing 10^{-3} M potassium ferrocyanide and 0.1 M potassium chloride.

Each electrode shows two peaks, one anodic and one cathodic, depending on the redox processes of the ferrocyanide ion on the surface of the working electrode. There can be seen well-defined and reversible peaks in all three cases.

The half-wave potential ($E_{1/2}$) is a qualitative characteristic for the analysed electroactive species and represents the value of the potential for which the current intensity is half of the maximum value [104]. In this case, this parameter has approximately the same value for CNF/C-SPE (0.163 V) and MWCNT/C-SPE (0.166 V) and a higher value for C-SPE (0.179 V). The I_c / I_a ratio is close to the ideal value 1 in all three cases, the closest value to the ideal one being obtained in the case of CNF/C-SPE (0.854 V). From these results it can be stated that CNF/C-SPE showed the highest sensitivity for the detection of ferrocyanide ion (I_a and I_c have the highest values). Also, CNF/C-SPE has the highest degree of reversibility, the difference between the potentials of the anodic and cathodic peaks (ΔE) is the smallest, and the I_c / I_a ratio has the highest value.

5.1.2. Calculation of the active surface of the electrodes

The electrode surface was determined by cyclic voltammetry, using the solution of 10^{-3} M potassium ferrocyanide and 10^{-1} M potassium chloride at scanning speeds between 0.1-1.0 V / s.

For CNF/C-SPE, a very good linearity was obtained between I_{pa} and the square root of the scanning speed (Figure 5.12. a)) with a coefficient of determination (R^2) of 0.9905. According to the literature, this result demonstrates that the electrochemical process at the working electrode is controlled by the diffusion of the electroactive species. Diffusion is the determining stage of speed.

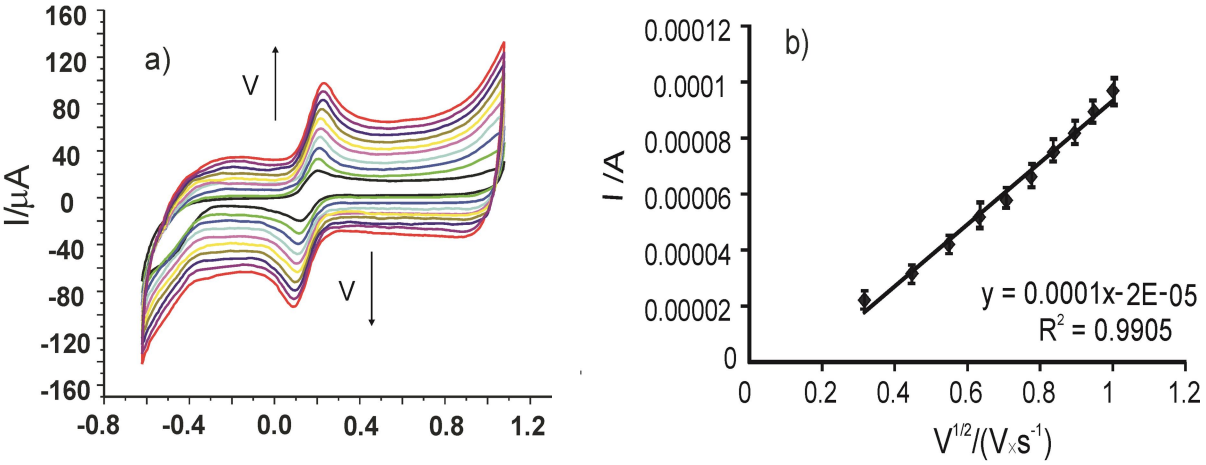

Figure 5.12. Variation of the electrochemical signal of CNF/C-SPE sensor at scan speeds between 0.1-1.0 V / s a); dependence of anodic current intensity on the square root of the scanning speed b)

The highest value of the active area was obtained for the CNF/C-SPE electrode (0.1524 cm^2), followed by MWCNT/C-SPE (0.0514 cm^2) and C-SPE (0.0500 cm^2). These results are consistent with the intensity of the peaks. From these values it follows that the sensitivity of the sensor decreases in order of CNF/C-SPE, MWCNT/C-SPE, C-SPE. This high sensitivity of CNF/C-SPE is due to the carbon nanofibers on the electrode surface, which by their small size but with a large specific area, favour the electron transfer and the selective accumulation or diffusion of analytes.

These results are consistent with the different studies published in the literature. This studies generally considers that CNF have excellent electrochemical sites superior to other nanomaterials, as they have very good conductivity and high chemical stability [82,105,106]. However, all three electrodes can be used successfully to determine caffeic acid, the phenolic compound of interest in this paper.

5.1.3. Electrochemical properties of electrodes in pure caffeic acid solution

Qualitative and quantitative determination of caffeic acid was carried out by cyclic voltammetry, which is a method that has many advantages (sensitivity, selectivity, simple equipment) and useful for interpretation of the processes that take place at the electrode surface. Cyclic voltammograms were recorded in 10^{-3} M caffeic acid solution, the support electrolyte solution being that of 0.1 M phosphate buffer (pH = 3.6). This pH value is optimal for the determination of caffeic acid with a very good sensitivity and selectivity [107]. It is necessary for the sensor responses to stabilize after five cycles in the optimized potential range (from -0.4 to 1.3 V). Thus, the results shown are those obtained after stabilization of voltammetric signal.

Figure 5.13. shows the cyclic voltammograms of all electrodes in 10^{-3} M caffeic acid solution (0.1 M phosphate buffer electrolyte, pH = 3.6).

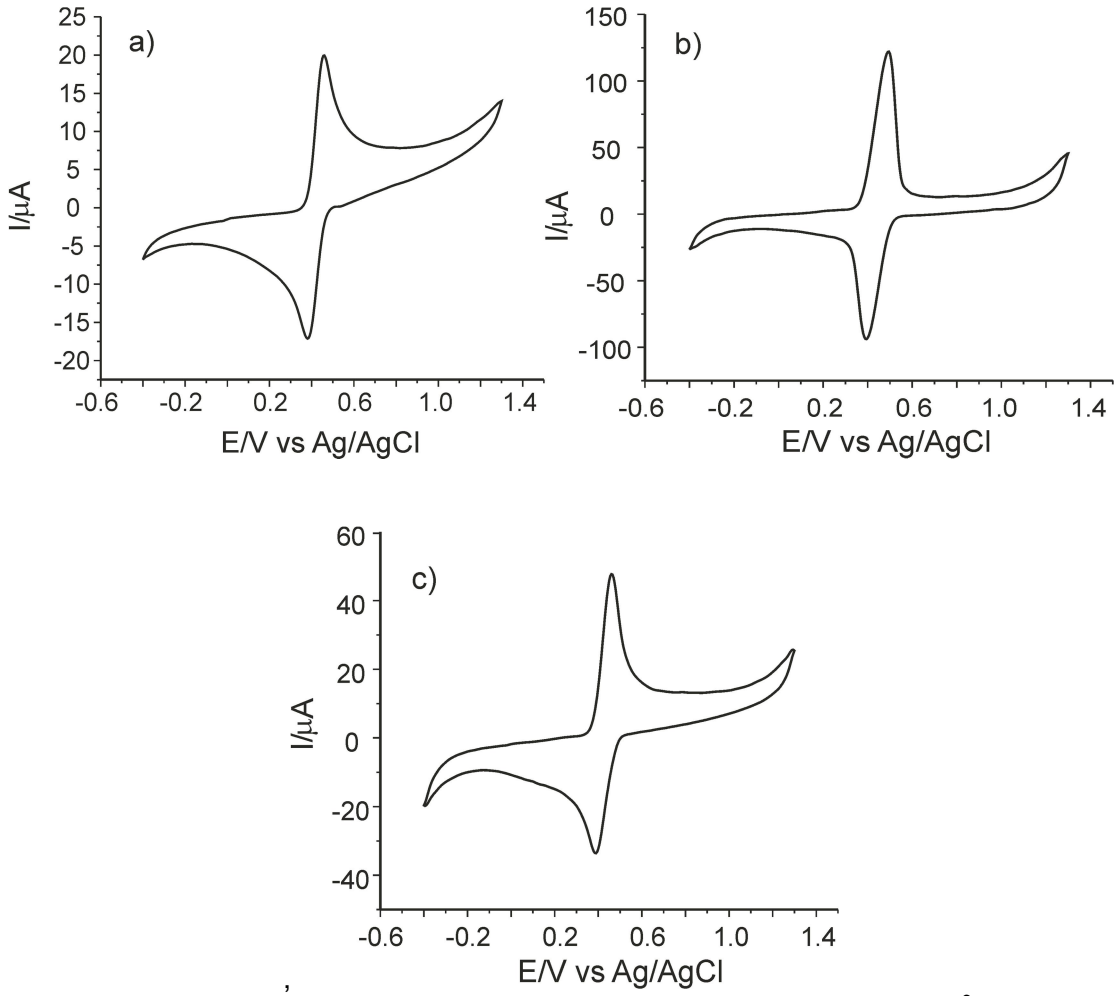

Figure 5.13. shows the cyclic voltammograms of all electrodes in 10^{-3} M caffeic acid solution (0.1 M phosphate buffer electrolyte, pH = 3.6).

An anodic and a cathodic peak were observed in the voltammogram, which correspond to the oxidation and reduction reactions of caffeic acid at the working electrode surface. According to the literature, redox processes consist in the oxidation of caffeic acid to the corresponding o-quinonic derivative, followed in the cathodic scan by the reduction of o-quinone to the initial compound. These processes involve the transfer of two electrons and two protons.

All the electrodes studied showed two clear and well-defined peaks and a quasi-reversible behaviour, as shown by the values ΔE and I_c / I_a. CNF-SPE has a reversible behaviour closer to ideal than MWCNT/C-SPE and C-SPE. CNF/C-SPE modified electrode shows the most intense anodic and cathodic peaks, demonstrating that it has better sensitivity to the detection of caffeic acid. The peaks observed in the case of MWCNT-SPE are more intense than in the case of C-SPE, which demonstrates the facilitation of electrochemical processes by the modifying nanomaterial. The sensitivity of the electrodes increases in the order: C-SPE <MWCNT/C-SPE <CNF/C-SPE.

5.1.4. Study of the kinetics and calculation of the diffusion coefficient of caffeic acid

To study the influence of the scanning speed on the voltammetric response, the cyclic voltammograms of CNF-SPE, CNT-SPE and C-SPE were recorded at different scanning speeds between 0.1 V / s and 1.0 V / s, increasing scan speed each time by 0.1 V / s.

To determine the factor that controls the kinetics of the electrode processes, the anodic peaks were plotted as a function of the scanning speed and the square root of the scanning speed.

Caffeic acid D values obtained with screen-printed electrodes are comparable to those obtained with other carbon-based electrodes reported in the literature [108–110],[111] It is known that carbon nanomaterials significantly improve the absorption of the analyte on the electrode surface, and also increase the electroactive surface of the sensor which accelerates the electrochemical reactions [59], [82,105,112].

These results prove that the screen-printed electrodes modified with carbon nanomaterials are sensitive and useful for the detection of caffeic acid.

5.1.5. Calibration curve and limit of detection

Calibration curves were performed for each sensor, C-SPE, CNF/C-SPE and MWCNT/C-SPE by recording cyclic voltammograms in solutions obtained from caffeic acid dissolved in 0.1 M phosphate buffer pH 3.6 of different concentrations between 0.1 and 40 μM. The scanning speed was 0.1 V / s, and the potential was within the range -0.4 V and 1.3 V.

In the case of the CNF / C-SPE sensor, the calibration curve, which represents the dependence between the anode peak current and the caffeic acid concentration (I_{pa} vs. c), is shown in Figure 5.19.

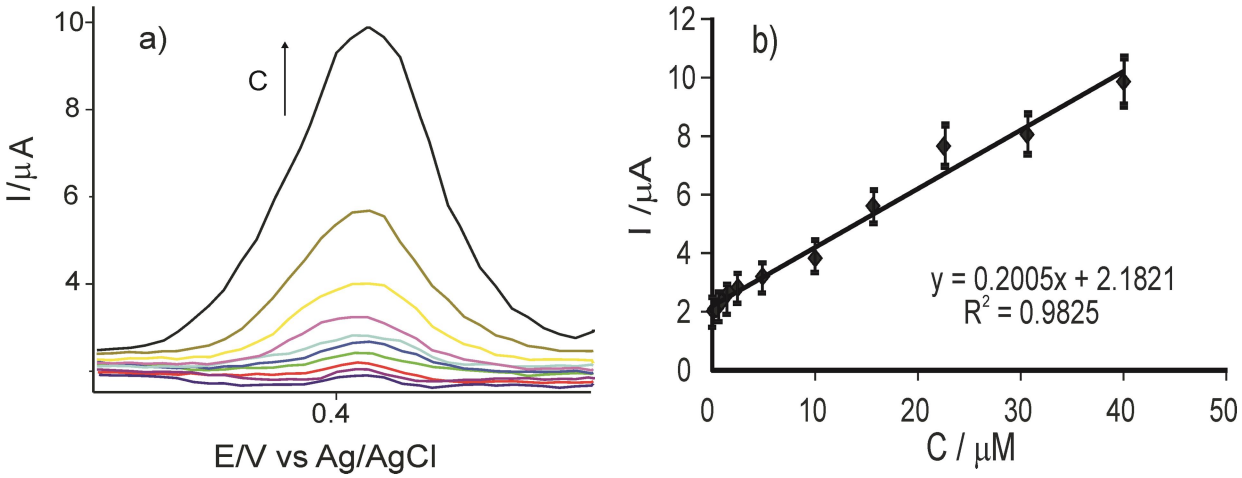

Figure 5.19. a) CV of CNF-SPE immersed in caffeic acid solution in the concentration range 0.1-40 μM (zoom of the anodic peak); b) Dependence of the anodic peak as a function of caffeic acid concentration.

There is a linear dependence between the intensity of the peak concentration in the range 0.1-40 and μM. The calibration line equation is I= 0,2005c + 2,1821.

Therefore, the calculation of LOD is carried out with the information collected from the first concentration used, where there is a linear dependence, and the correlation coefficient is closer to one.

The results obtained for the detection limit and the quantification limit for the three sensors are presented in Table 5.8.

Table 5.8. LOD and LOQ values of the three sensors for caffeic acid detection

Sensor	LOD (M)	LOQ (M)
C-SPE	1.27×10^{-7}	4.23×10^{-7}
CNF/C-SPE	3.23×10^{-9}	1.077×10^{-8}
MWCNT/C-SPE	6.1×10^{-8}	2.02×10^{-7}

The low values of LOD and LOQ are lower or comparable to those reported in the literature. This is due to the high sensitivity of the sensors tested in this study, demonstrating the feasibility of the method. All three sensors can be used successfully in the analysis of real samples for AC detection, but CNF / C-SPE has the lowest limits of detection and quantification. The following analyses will determine the amount of caffeic acid in food supplements using the three electrodes studied so far.

5.1.6. Study of the determination of caffeic acid in food supplements using C-SPE, CNF / C-SPE and MWCNT / C-SPE

C-SPE, CNF / C-SPE and MWCNT / C-SPE were used to determine caffeic acid in three non-standardised food supplements (Active Detox-Herbagetica, DVR-Stem Glicemo-DVR Pharm and Green Tea-Alevia), comparing the results with those obtained by the spectrophotometric method. The measurements were recorded by cyclic voltammetry in the potential range between -0.4 V and 1.3 V with different scanning speeds between 0.1-1.0 V / s.

Analysing each sample, a pair of redox peaks is observed related to the presence of caffeic acid. In each case, less defined peaks are observed, which correspond to other active compounds found in these dietary supplements. The intensities of the anodic and cathodic peaks increase with increasing of scanning speed in each situation. Plotting the intensity of the anodic peak as function of scanning speed, a linear dependence with $v^{1/2}$ is obtained, which demonstrates that the electrochemical process is controlled by diffusion, in each case.

The CNF / C-SPE sensor demonstrated a better detection limit, higher intensity peaks and better defined, but also a lower background noise, compared to C-SPE and MWCNT/C-SPE. Therefore, the quantification of caffeic acid in the analysed food supplements will be performed using the calibration line obtained by CNF/C-SPE. The results will be compared with those obtained by the Folin-Ciocalteu spectrophotometric method. Stability and interference studies will also be performed for CNF/C-SPE.

5.1.7. Validation of the voltammetric method

The Folin-Ciocalteu spectrophotometric method was used to validate the new electroanalytical method based on the modified C-SPE. The standard solutions were solutions prepared with caffeic acid of different concentrations [113].

The content of one capsule of each food supplement was dispersed in 50 mL of PBS solution (pH = 3.6) for electrochemical analysis. Ultrasonication followed by filtration was used to properly prepare the samples for analysis.

The amounts of caffeic acid in food supplements were calculated from the calibration lines corresponding to the two methods of determination, spectrophotometric and voltammetric (CNF / C-SPE), considering dilutions and the amount of food supplement taken in the analysis. The results, expressed in mg caffeic acid per dose, are given in Table 5.12.

Table 5.12. Caffeic acid content determined in the samples analysed by the spectrophotometric and voltammetric methods

Dietary supplement	Voltammetric method Caffeic acid (mg / capsule)	Spectrophotometric method Caffeic acid (mg / capsule)
Detox-Activ	101.5 ± 4.1	102.4 ± 3.9
DVR Stem Glicemo	190.5 ± 7.2	202.1 ± 8.1
Ceai Verde (Green Tea)	177.6 ± 5.6	177.7 ± 4.8

The caffeic acid content values obtained by the two methods are similar. Analysis of variance (ANOVA) showed that there is a significant difference between the three sets of values at a confidence level of 95%. This demonstrates that the voltammetric method is a sensitive, valid and accurate method for determining caffeic acid in food supplements.

In the next step the polyphenol content was determined by the UHPLC-MS / MS method with ESI ionization, using a high-resolution Q Exactive mass spectrometer ™ Focus Hybrid Quadrupole–OrbiTrap (ThermoFisher Scientific) equipped with HESI, coupled to a liquid chromatograph high performance UltiMate 3000 UHPLC (ThermoFisher Scientific).

Calibration was performed for each of the phenolic acids and flavonoids in the range of 50 - 2000 µg / L, by diluting in series with methanol the standard mixture of concentration 10 mg/L. From the data obtained, the amount of caffeic acid present per capsule was calculated for each product. The results are found in Table 5.14.

Table 5.14. Caffeic acid analyzed by HPLC method

Dietary supplement	HPLC method Acid cafeic (mg / capsule)
Detox-Activ	99.84
DVR Stem Glicemo	197.9
Ceai Verde (Green Tea)	185.58

From Table 5.14. the values obtained by chromatographic method are close to those obtained by CV and IR, which proves once again, the accuracy, selectivity and sensitivity of the CNF / C-SPE sensor and the feasibility of the voltammetric method.

5.1.8. Stability studies

The CNF / C-SPE sensor may be used repeatedly under the measurement conditions of this study, without changing its cyclic voltammogram by more than 5% for at least 100 measurements. However, the relative standard deviation (RSD) of 10 replicated cyclic voltammograms recorded in the 10^{-5} M caffeic acid solution is 3.02%. The contact time with the solution to be analysed should be as short as possible and the sensor should be washed with electrolyte support solution (PBS pH 3.6 in this case) and ultrapure water after each measurement.

5.1.9. Interference studies

Some chemically related species were studied to evaluate the selectivity of the CNF / C-SPE sensor for the detection of caffeic acid in complex samples, which could interfere with the quantification of 10^{-5} M caffeic acid under optimal conditions.

The determination of caffeic acid is more influenced by the presence of catechol, while gallic acid, L-ascorbic acid, uric acid, ferulic acid and vanillic acid have little influence in the quantitative determination of caffeic acid. This is related to the oxidation potentials of catechol and gallic acid, which are close to that of caffeic acid.

The other interfering compounds appear in cyclic voltammograms as new peaks, which do not influence the peaks of caffeic acid. From these results, it can be concluded that the CNF / C-SPE sensor is selective for caffeic acid in real samples and has good accuracy and selectivity.

5.2. Conclusions

Following the analysis of electrodes immersed in a double solution of potassium ferrocyanide and potassium chloride, well-defined and reproducible peaks were recorded, which can be used for the analysis of real samples. There can be observed similar characteristics of the three electrodes in the analysis of the pure caffeic acid solution. This proves that all three sensors can give satisfactory results.

Subsequently, the diffusion coefficient of caffeic acid was calculated for each electrode. A better result was obtained when using CNF / C-SPE. After this analysis, the three sensors were used to plot the calibration curves, useful for further analysis. There is a linear dependence between the peak intensity and the concentration of the solution in the chosed range (0.1-40 μM) for all three sensors used.

At higher concentrations, the electrochemical signal increases more slowly, due to the saturation of the active centres on the working electrode surface. The detection limits have low values, which proves once again that the sensors have a good sensitivity. The lowest LOD value was obtained for CNF / C-SPE. In the last part of the paper, the detection of caffeic acid in three food supplements (Active Detox, DVR Stem Glycemo, Green Tea) was performed.

In all three cases, the characteristic peaks of the substances analysed were highlighted, peaks that correspond to the redox reactions at the level of the working electrode. Being complex pharmaceutical formulations, in addition to the characteristic peaks of the existing caffeic acid, there were also peaks due to excipients or other electroactive pharmacological principles, present in the sample to be analysed. Both the Folin Ciocalteu

spectrophotometric method and the HPLC method were used to validate the results, through which similar results were obtained.

Thus, it has been shown that the voltammetric method is useful and valid for determining the caffeic acid content of food supplements. The analysed sample requires minimal treatment before analysis, and the equipment is simple, portable and sensitive. This analytical method is useful as a screening method to determine the caffeic acid content of nutritional supplements or pharmaceutical samples. The small amount of sample required, the reduced time and portability are the advantages that make this electroanalytical method suitable for quality control of food supplements.

In the future, nanotechnology will be used more frequently to modify sensors with nanomaterials to increase the detection properties.

CHAPTER 6. Development of screen-printed sensors and biosensors for ferulic acid determination in cosmetics

Introduction

The aim of this paper is both to study the electrochemical behaviour and the qualitative and quantitative determination of ferulic acid with the three types of electrodes: screen-printed electrode based on carbon nanofibers (CNF / SPE), screen-printed electrode based on carbon nanofibers modified with gold nanoparticles (CNF-GNP / SPE) and a biosensor obtained from the modification of CNF-GNP / SPE by tyrosinase immobilizing on its surface. Also, the electroanalytical method was validated for the quantification of ferulic acid in various cosmetics using a classical method, infrared spectrometry.

6.1. Results and discussions

6.1.1. Voltammetric behaviour of electrodes in PBS and potassium ferrocyanide

The preliminary analysis evaluated the electrochemical behaviour of CNF / SPE, CNF-GNP / SPE and CNF-GNP-Ty / SPE. The electrolytic solution used was a phosphate buffer solution (PBS pH 7.0), and potassium ferrocyanide solution of 10^{-3} M and 10^{-1} M PBS.

The first potential range used was -1.0 V and +1.3 V, in which the signal was not stable with any of the electrodes. Thus, gradually the potential of the negative vertex was increased, until a stable signal was obtained, both in the case of PBS solution, potassium ferrocyanide-PBS and ferulic acid-PBS. There was obtained a stable signal for the analysis of all solutions at a potential ranged between -0.4 and +1.3 V.

Because in the potential range -1.0 V and +1.3 V the signal was not stable with any of the electrodes, the potential of the negative vertex was increased, until a stable signal was obtained, both in the case of the PBS solution, ferrocyanide of potassium-PBS and ferulic acid-PBS. The stable signal for all analysed solutions was observed in the potential range between -0.4 and +1.3 V.

This potential domain was initially used to study the electrochemical behaviour of CNF / SPE, CNF-GNP / SPE and CNF-GNP-Ty / SPE immersed in 10^{-1} M PBS (pH 7.0). The scan speed was 0.1 V × s^{-1}. A cathodic peak was observed in the case of CNF-GNP / SPE and CNF-GNP-Ty / SPE at E = 0.440 V (current -13.361 μA) and E = 0.455 V respectively (current -11.286 μA). These peaks occur due to the modification of the screen-printed electrodes with gold nanoparticles, respectively tyrosinase, in the case of the biosensor. The background current was reduced for all three electrodes used.

The following analysis was performed by immersing the three electrodes in a solution of 10^{-3} M potassium ferrocyanide 10^{-1} M-PBS (pH 7.0). The potential range was between -0.4 and +1.3 V, and the scan speed of 0.1 V × s^{-1}. In the cyclic voltammograms obtained with the three electrodes, were observed an anodic peak and a cathodic peak. These peaks are due to the process of oxidation-reduction of ferrocyanide that takes place at the surface of the electrode.

Following the recorded and calculated parameters it can be stated that $E_{1/2}$ has close values for CNF / SPE and CNF-GNP / SPE and a lower value for CNF-GNP-Ty / SPE. All three electrodes have an I_{pc}/I_{pa} ratio that exceeds the value 1. From the I_{pc}/I_{pa} and ΔE values, higher than the theoretical values, it results that the redox processes are quasi-reversible [114].

The highest peaks observed are in the case of CNF-GNP / SPE, which shows a higher sensitivity to the detection of potassium ferrocyanide and the synergistic effect of gold nanoparticles in electrodetection. The presence of Ty on the biosensor surface is clearly highlighted by the differences observed between the electrochemical behaviour of CNF-GNP / SPE and that of CNF-GNP-Ty / SPE.

6.1.2. Electrodes active surface area

In the next stage cyclic voltammograms were recorded at different scan rates (0.1-1.0 $V \times s^{-1}$) using potassium ferrocyanide solution 10^{-3} M, 10^{-1} M PBS (pH 7.0).

For all electrodes, there is a linear dependence between the anodic peak current and the square root of the scanning speed, which demonstrates that the electrochemical process is controlled by the diffusion of electroactive species [115].To calculate the active area of the electrodes was used Randles-Sevcik equation [116].

The results obtained are presented in Table 6.3.

Table 6.3. Active surface area of the electrodes used in the assay

Electrode	Surface active area (cm^2)
CNF/SPE	0.1819 ± 0.0036
CNF-GNP/SPE	0.1868 ± 0.0037
CNF-GNP-Ty/SPE	0.1774 ± 0.0035

The CNF-GNP-Ty / SPE biosensor has the lowest value of the active surface because the Ty immobilized on the electrode surface does not participate in the oxidation-reduction process of ferrocyanide, demonstrating selectivity. The two screen-printed sensors have a close active surface in value, but the modification of the electrode surface with gold nanoparticles explains the larger active area of CNF-GNP / SPE.

6.1.3. The voltammetric response of the electrodes in the ferulic acid solution

CNF-GNP / SPE and CNF-GNP-Ty / SPE electrodes were used for ferulic acid detection studies with higher sensitivity and better selectivity. Figure 6.6 shows, by comparison, the obvious response of the two electrodes, when immersed in a solution of 10^{-3} M ferulic acid - 10^{-1} M PBS (pH 7.0) b).

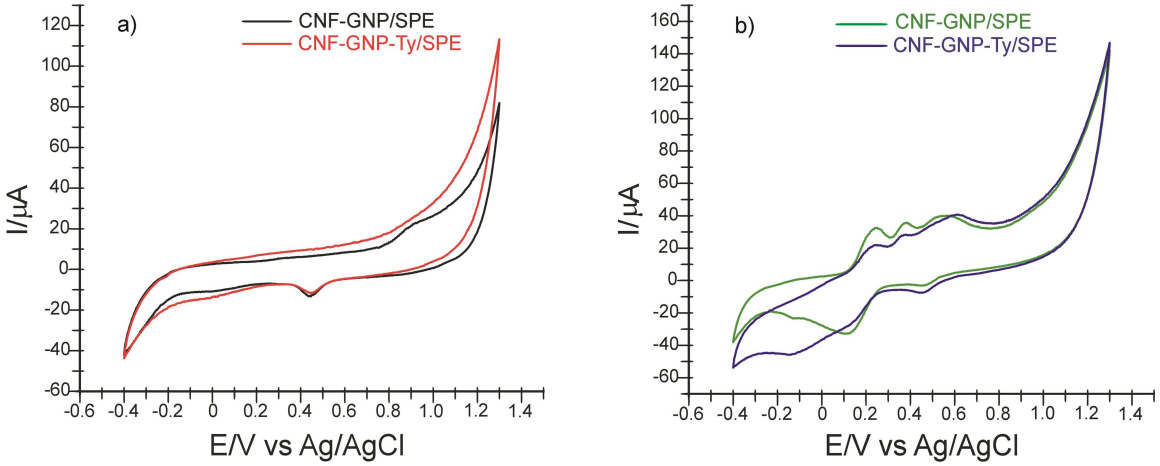

Figure 6.6. a) Cyclic voltammograms of CNF-GNP / SPE (black line) and CNF-GNP-Ty/SPE (red line) immersed in 10^{-1} M PBS solution (pH 7.0). b) Cyclic voltammograms of CNF-GNP / SPE (green line) and CNF-GNP-Ty / SPE (blue line) immersed in 10^{-3} M ferulic acid solution and 10^{-1} M PBS (pH 7.0). Scan speed: 0.1 $V \cdot s^{-1}$.

In the presence of ferulic acid, there are three anodic and two cathodic peaks of different intensities and potentials, related to the oxidation and reduction of ferulic acid at the level of the sensitive element. This electrochemical behaviour is like that observed in other previously published studies [117].

In the case of CNF-GNP-Ty / SPE the cathodic peak potential has a lower value. This shift to negative potential values indicates that the reduction process is strongly influenced by the presence of the enzyme [118,119]. This detection at a lower potential indicates that the reduction process needs a lower activation energy in the case of the biosensor [120].

Therefore, the biosensor has better sensitivity and selectivity compared with the gold nanoparticles modified sensor in detection of AF. The reduction processes are those of the quinone compounds formed by anodic oxidation, processes that take place in two stages resulting in two well-defined cathodic peaks.

Enzyme immobilization was confirmed by voltammetric analysis, as seen in Figure 6.6. It is found that the enzyme immobilized in the biosensor catalyses the reactions of hydroxylation of the benzene nucleus and oxidation of the ortho-biphenolic derivative to the corresponding quinone [121]. For this reason, the reduction peak is substantially modified, being the main difference observed between the sensor and the biosensor.

Obtaining lower peak potential values suggests a rapid process of electron transfer in the redox process of ferulic acid at the active surface [122]. When analysing the signal obtained with CNF-GNP-Ty / SPE, the cathodic peak potential has a much lower value than in the case of CNF-GNP / SPE, which means that the process of reducing the electrochemical oxidation of ferulic acid requires a lower activation energy in the case of the biosensor [123]. In addition, the cathodic current value of CNF-GNP-Ty / SPE is higher than in the case of CNF-GNP / SPE, which demonstrates that the biosensor is more sensitive to the electrochemical detection of the ferric acid oxidation product. Also, the I_{pc}/I_{pa} ratio is higher in the case of the biosensor. This increase of cathodic current in the case of the biosensor is due to tyrosinase which catalyses the oxidation process of ferulic acid [124].

Consequently, in the case of CNF-GNP-Ty / SPE, the oxidation of ferulic acid takes place through a mechanism involving the transfer of two protons and two electrons [125]. Following the oxidation of ferulic acid, the main product obtained is the o-quinonic derivative of ferulic acid [125].The tyrosinase immobilized on the sensor surface increases the selectivity of the biosensor, a fact confirmed by the increase of the cathodic peak current and the displacement of the cathodic peak to a more negative potential compared to that observed for CNF-GNP / SPE.

In the next step, the electrochemical behaviour of the two electrodes was followed at different speeds (in the range between 0.1 V × s^{-1} and 1.0 V × s^{-1}), increasing the scanning rate each time by 0,1 V × s^{-1}, in 10^{-3} M ferulic acid solution (the supporting electrolyte was 10^{-1} M PBS solution at pH 7.0). The linear dependence between I_c and v confirms that the redox process of ferulic acid is controlled by the electron transfer process [126]. Therefore the reduction process is governed by Laviron's equation [127].

Comparing the results obtained with CNF-GNP-Ty / SPE and CNF-GNP / SPE, it can be stated that in both cases the reduction process is controlled by electron transfer, the process being faster in the case of the biosensor (comparing the slopes of the two adjustment equations presented in Table 6.5.). Using the Laviron equation, the values of the concentration of the oxidized species (Γ) were calculated, and the results are presented in Table 6.5.

Table 6.5. The linear equation of the line (I_c vs. v), R^2 and Γ.

Electrode	Line equation	R^2	Γ (mol·cm^{-2})
CNF-GNP/SPE	$y = -2.585 \times 10^{-5} x - 2.348 \times 10^{-5}$	0.9994	5.02×10^{-11}
CNF-GNP-Ty/SPE	$y = -3.1137 \times 10^{-5} x - 3.4962 \times 10^{-5}$	0.9996	6.05×10^{-11}

$y = I_{pc}$; $x = v$.

From these results it can be appreciated that the biosensor has superior electroanalytical properties to the detection of ferulic acid. In addition, the presence of tyrosinase ensures superior selectivity of the biosensor in complex samples. Immobilization of tyrosinase together with carbon nanofibers and gold nanoparticles leads to better biosensitivity

and conductivity, these nanomaterials having a synergistic effect in biodetection. Therefore, the biosensor was used for quantitative analyses.

6.1.4. The biosensor response to different concentrations of ferulic acid

Subsequently, cyclic voltammetry was used to detect ferulic acid of various concentrations using CNF-GNP-Ty / SPE. It is observed that the intensity of the reduction peak I increases with the increase of the ferulic acid concentration in the studied concentration range, from 0.1 to 129.6 μM (Figure 6.9.)

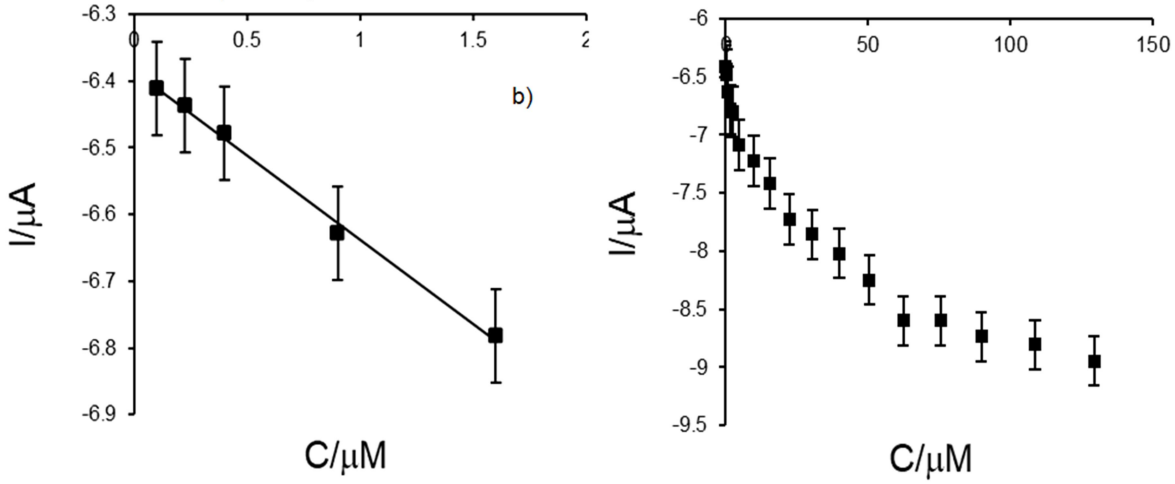

Figure 6.9. Biosensor calibration curve in the concentration range 0.1–129.6 μM a) and 0.1–1.6 μM b).

The increase of the reduction current is linear with the concentration in the range from 0.1 to 1.6 μM, and the linear regression equation is $y = -0.2529x - 6.3845$ ($R^2 = 0.9961$, $n = 5$) with a limit of detection (LOD) of 2.89×10^{-9} mol L^{-1} and a limit of quantification (LOQ) of 9.64×10^{-9} mol L^{-1}.

6.1.5. Stability, reproducibility, repeatability. Interference studies

The biosensor is stable and can be used for more than 50 measurements by cyclic voltammetry in solutions containing FA. As regards the reproducibility of the manufacturing method, no more than 2 % differences have been obtained between identically prepared biosensors immersed in FA solutions of the same concentration. Also, the variation of the response of the biosensor when determining the FA in solution of the same concentration, when removing from the solution, rinsing and repeating the cyclic voltammogram did not exceed 3 %. The biosensor has proven a very good selectivity, the potential and current of the cathodic peak remaining practically unchanged at the additions of compounds that are found in cosmetics, for example propanediol, glycerine, vitamin E, etc.

6.1.6. Determination of FA in cosmetics

To verify the practicability and feasibility of the proposed method, CNF-GNP-Ty/SPE was used for the detection of ferulic acid in cosmetics with different presentation forms and consistency: serum, cream, and emulsion.

Figure 6.11. shows the cyclic voltammograms of CNF-GNP-Ty / SPE immersed in ordinary antioxidant serum solutions of different concentrations. The representative peaks of the electrochemical processes of the ferulic acid can be observed, and the reduction peak I was used for quantification.

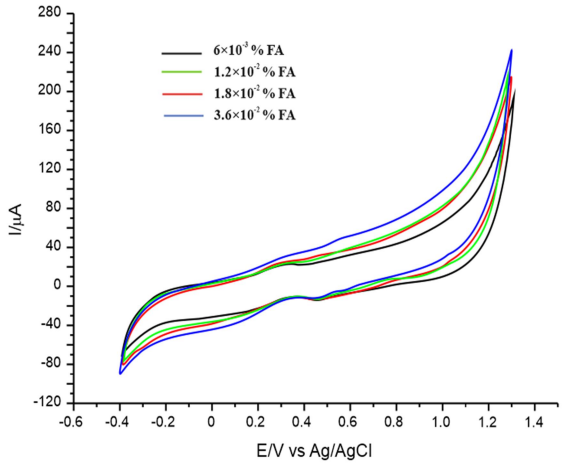

Figure 6.11. Cyclic voltammograms of CNF-GNP-Ty / SPE immersed in solutions obtained from the antioxidant serum Ordinary of different concentrations: 6 × 10^{-3}% FA (black line); 1.2 × 10^{-2}% FA (green line), 1.8 × 10^{-2}% FA (red line), 3.6 × 10^{-2}% FA (blue line).

Considering the current of the cathodic peak, the quantity of cosmetic product taken into analysis and the equation of the calibration line, the concentration of ferulic acid in the cosmetic products was calculated, obtaining the results included in Table 6.6.

To validate the voltammetric method, cosmetics were also analysed by the infrared spectrometric method.

The amounts of ferulic acid in cosmetics were calculated from the calibration equation corresponding to the peak at 1050 cm^{-1}, a bit related to the elongation vibration (stretch) of the phenolic CO group [128]. All experiments were performed in triplicate and the results are presented in Table 6.6.

Table 6.6. Results of quantification of ferulic acid in cosmetics

Cosmetic product	c% FA FTIR Method	c% FA Voltammetric method
ORDINARY	2.932	3.114
EMULSIE	0.090	0.104
SABIO	0.096	0.112

RSD= 2%

Close values of AF concentration in cosmetics are obtained by the two methods, which demonstrates that the CNF-GNP-Ty / SPE biosensor method is useful for quantifying AF with adequate accuracy.

In the case of the ORDINARY product, in which the manufacturer indicates a concentration of 3% FA, the results obtained by both methods are close to those indicated, demonstrating the accuracy and precision of the two methods in the detection of FA. For the separation, identification, and quantification of ferulic acid from cosmetic samples, high performance liquid chromatography was used together with electrospray ionized mass spectrometry.

From the data obtained, the concentration of ferulic acid was calculated for each product. The results can be found in Table 6.9.

Table 6.9. Ferulic acid content determined in the samples analyzed by the UHPLC method

Dietary supplement	UHPLC method Ferulic acid %c
ORDYNARY	3.147
GEROVITAL	0.082
SABIO	0.0504

From the results obtained by quantifying FA using UHPLC, we can see that the values are closer to those obtained by the voltammetric method in the case of Ordinary antioxidant serum and Gerovital cleansing emulsion.

In the case of the Sabio product there is a greater difference between the values. This difference could be caused by the influence of the other phenolic compounds with similar structure, existing in the sample.

6.2. Conclusions

Three (bio) sensors based on nanomaterials and tyrosinase for the electrochemical detection of ferulic acid were developed and characterized. The CNF-GNP-Ty / SPE biosensor was constructed by the drop-and-dry technique, followed by crosslinking tyrosinase using glutaraldehyde. CNF-GNP-Ty / SPE has been shown to be useful in the analysis of ferulic acid in cosmetics. Quantification based on the cathodic peak allowed the selective detection of AF from complex matrices. The use of cyclic voltammetry as a detection method allowed the study of FA detection and the achievement of excellent analytical performance with applicability in electroanalysis.

The results obtained with the CNF-GNP-Ty / SPE biosensor are are close to those obtained by the standard FTIR analysis method or those indicated by the manufacturer. UHPLC analysis also showed similar amounts of ferulic acid as the voltammetric method.

The method performed in this study has a number of advantages, such as simplicity and low cost. In addition, the method has a very good accuracy, is versatile and can be used in routine analysis in the quality control of cosmetics, pharmaceuticals, food supplements and other types of samples.

CHAPTER 7. Development of new electrochemical biosensor based on carbon nanofibers, cobalt phthalocyanine and laccase for the detection of p-coumaric acid in phytopreparations

In the present paper, we chosen a screen-printed electrode based on carbon nanofibers (CNF / SPE) for the simultaneous quantification of two hydroxycinnamic acids (caffeic acid and ferulic acid) from a product with a complex formula, phyto-homeopathic, with the role in reducing the occurrence of visual disturbances. CNF / SPE was chosen based on previous studies, which demonstrated a high sensitivity and reproducibility. In this study CNF / SPE has been shown to be appropriate and effective, with a low detection limit for the determination of both electroactive compounds. The electrochemical technique used was cyclic voltammetry.

The aim was to determine the antioxidant capacity of these compounds in the Eye Blend product by the DPPH method.

7.1. Results and discussions

7.1.1. Preliminary studies for electrode characterization

Preliminary studies characterizing the CNF / SPE sensor were performed both in PBS solution pH = 7.0 and in 10^{-3} M potassium ferrocyanide solution and described in detail in the previous paper. Following the calculations, the area of the active surface of the sensor has the value 0.1819 cm^2, being superior to the geometric area, which shows an optimal sensitivity of the sensor.

7.1.2. Voltammetric behaviour of CNF / SPE in ferulic acid and caffeic acid solutions

In the next step, were analysed the solutions of ferulic acid, respectively 10^{-3} M caffeic acid (10^{-1} M PBS support electrolyte, pH 7.0) with the CNF / SPE sensor, using cyclic voltammetry.

In Figure 7.1. the cyclic voltammogram obtained by CNF / SPE by immersion in 10^{-3} M ferulic acid solution (PBS electrolyte pH = 7.0) is presented at the second scan. Three anodic peaks and two well-highlighted cathodic peaks can be observed.

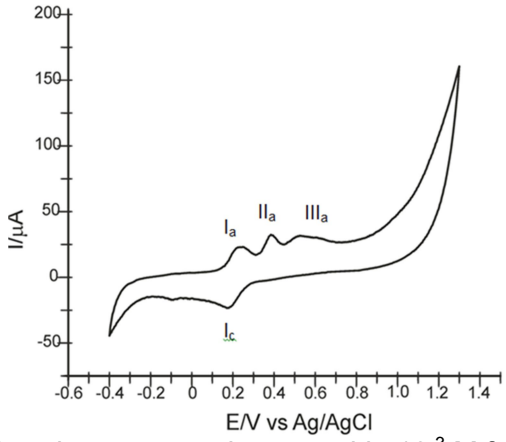

Figure 7.1. CNF / SPE cyclic voltammogram immersed in 10^{-3} M ferulic acid solution and 10^{-1} M PBS (second scan) at 0.1 V / s

Initially, the recorded cyclic voltammogram showed two irreversible oxidation peaks II_a at E = 0.388 V and III_a at E = 0.552 V, corresponding to the beginning of the electro-polymerization process. The value of the measured potential is close to that obtained in other studies [125]. On the second scan (Figure 7.1.), peaks II_a and III_a decrease in intensity and the pair of peaks, I_a/I_c, is highlighted, indicating the deposition of an electroactive film on the electrode surface.

The two peaks (I_a = 23.308 μA, E = 0.235 V) / I_c (I_c = -23.87 μA, E = 0.179 V), correspond to a quasi-reversible redox process (the oxidation-reduction mechanism of the o-quinone / o-hydroquinone). An additional II_c peak can be observed without an anodic counterpart.

Peaks II_a and III_a are related to the oxidation of FA, eliminating an electron and a proton, leading to a stable radical, followed by a second elimination of electrons, by cleavage of the methoxy group, which leads to the formation of o-quinone, a reaction that explains the reversible system I_c / I_a [125].

The electrochemical behaviour of CNF / SPE in a 0.1 M caffeic acid solution and 0.1 M PBS pH 7.0 was also studied. In Figure 7.2. an anodic and a cathodic peak are observed, related to the oxidation-reduction process of caffeic acid. The mechanism has been explained in detail in section 5.2.3. The anodic peak is recorded at E = 0.242 V with I_{pa} = 64.055 μA and the cathodic peak at E = 0.160 V and I_{pc} = -36.461 μA.

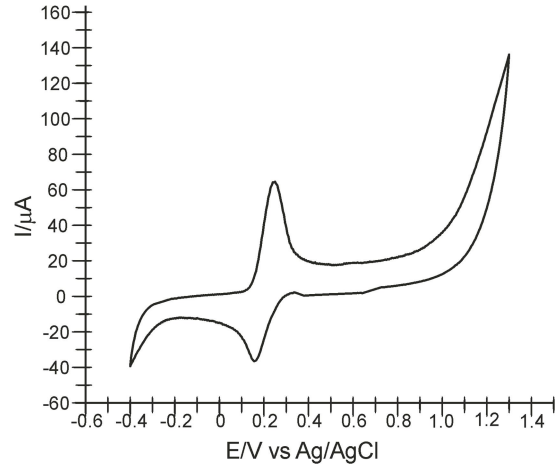

Figure 7.2. Cyclic voltammogram of CNF / SPE immersed in 10^{-3} caffeic acid solution

Next, the influence of the scanning rate on the voltammetric response of the CNF / SPE sensor in AC solution, respectively FA 10^{-3} M (electrolyte support PBS 0.1 M pH 7.0), at

different scan rates between 0.1 V / s and 1.0 V / s, increasing the scanning speed each time by 0.1 V / s.

Following the graphical representation of the anode peak currents as a function of the square root of the scanning speed, a linear dependence was found between the two parameters, which demonstrates that the redox process is controlled by the diffusion phenomenon.

The results obtained are presented in Table7.1.: 10^{-3} M caffeic acid solution (PBS solution 10^{-1} M pH = 7.0) at 0.1 V / s.

Table 7.1. Linear equations, R^2 and diffusion coefficients of CA and FA

Phenolic compound	Linear equation	R^2	D ($cm^2 \times s^{-1}$)
CA	$y = 1.793\times10^{-5} - 3.266\times10^{-5}$	0.9969	3.48×10^{-6}
FA	$y = 2.725\times10^{-5} - 2.942\times10^{-5}$	0.9999	7.24×10^{-6}

7.1.3. The influence of AF and CA concentration on the electrochemical response

The following experiments focus on the simultaneous determination of the two hydroxycinnamic acids using CNF / SPE. For this, 10^{-3} M solutions of AC and FA were used. For each case a calibration curve related to the other phenolic compound added in the concentration range 10-1000 μM was performed.

Cyclic voltammograms obtained by immersion of CNF / SPE in a 10^{-3} M caffeic acid solution (PBS support electrolyte pH = 7.0) containing increasing concentrations of ferulic acid are shown in Figure 7.5.

The values of the diffusion coefficients of CA and FA are lower than other values obtained in the literature [30,129–131].

Also, for caffeic acid, there is a slight difference between the value obtained now, compared to that obtained in the previous study (4.13×10^{-6} $cm^2 \times s^{-1}$), although the same type of electrode was used. These differences in the value of D could be explained using the electrolyte solution with a higher pH [108].

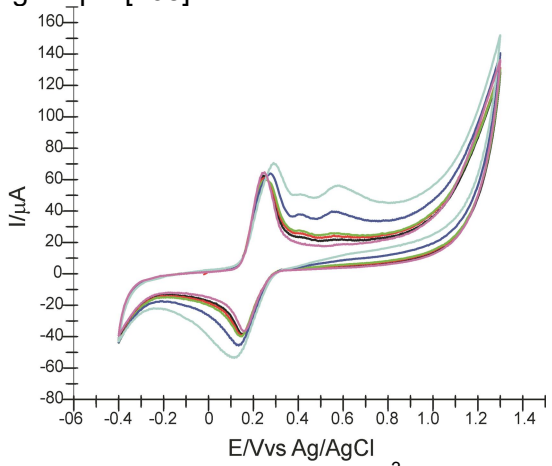

Figure 7.5. Cyclic voltammograms of CNF / SPE in 10^{-3} M caffeic acid solution (purple line), then containing different concentrations of ferulic acid: 10 μM (black line), 50 μM (red line), 100 μM (green line), 500 μM (blue line) and 1000 μM (blue line).

An anodic and a cathodic peak of higher intensities can be observed, corresponding to the presence of caffeic acid, and as the increasing amounts of ferulic acid are added, the irreversible peaks II_a and III_a related to FA electropolymerization are better highlighted. The deposition of FA on the surface of the electrode that would correspond to a pair of I_a/I_c peaks overlaps with the corresponding peaks the oxidation-reduction process of CA.

When the first concentrations of AF are added, the first anodic peak undergoes a slight decrease in intensity, which means that the oxidation process of CA is inhibited by the deposition and oxidation of FA. In contrast, when the concentration of added FA in the solution is equal to the concentration of AC, both the intensity of the anodic peak (I_{pa} = 70.394 μA) and

the potential (E_a = 0.287V) have higher values. This can be explained by the fact that FA is more electrochemically active than CA.

Compared to the anodic peak, the cathodic peak increases in intensity proportional to the increase of the FA concentration in the solution, simultaneously with the displacement of the potential towards lower values .This indicates that both compounds simultaneously undergo a process of reduction.

Figure 7.6. shows the cyclic voltammograms obtained by CNF / SPE recorded in a solution of 10^{-3} M FA, a concentration that remains constant, increasing exponentially the AC concentration (10-1000 μM). As the concentration of CA in the solution increases, a higher intensity of the first anodic peak, related to the oxidation of caffeic acid, is highlighted. The presence of CA in solution does not prevent oxidation of FA. The constant increase in the intensity of the anodic peak is explained by the synergy of the oxidation processes of the two compounds.

When in the solution we have the same concentration of AC and FA, the potential of the first anodic peak moves to a higher value. Regarding the cathodic peak, when in the solution with a constant concentration of AF, amounts of CA are progressively added, the intensity of the peak increases at a faster rate, and the potential shifts to lower values.

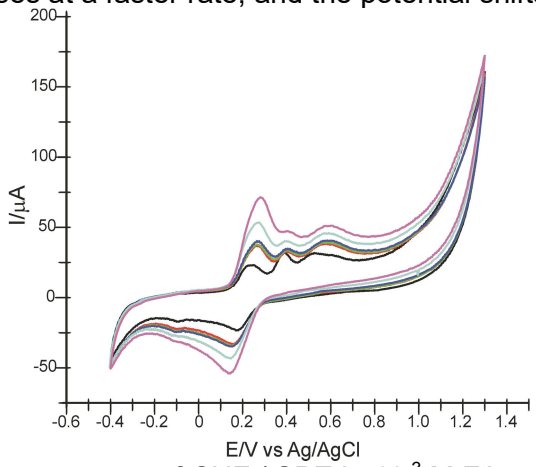

Figure 7.6. Cyclic voltammograms of CNF / SPE in 10^{-3} M FA solution (black line), then containing different AC concentrations: 10 μM (red line), 50 μM (green line), 100 μM (blue line), 500 μM (blue line) and 1000 μM (purple line).

Based on the cyclic voltammograms obtained above, was studied the relationship between the added concentrations and the intensity of the cathodic peaks for CA and FA. The cathodic peaks had a constant increase in both situations, which showed that the reduction process was influenced by the presence of both antioxidants. The concentration range studied was 10-1000 μM for the studied phenolic compound, while the concentration of the other was constant.

There is a linear dependence between the concentration of FA (a) and CA (b), respectively, and the intensity of the recorded cathodic peak. The equations of the calibration plotts, but also the LOD and LOQ values obtained are noted in Table 7.2.

Table 7.2. LOD and LOQ values, line equation and R^2 of the CNF / SPE sensor for simultaneous detection of AC and FA

CNF/SPE	LOD (M)	LOQ (M)	R^2	The equation of the line
FA Detection	$2.33x10^{-7}$	$7.78x10^{-7}$	0.9961	I(μA) = -0.0143 μg / μL – 38.436
CA Detection	$2.39x10^{-7}$	$7.97x10^{-7}$	0.9981	I(μA)= -0.0205 μg / μL - 33.148

The low values of the limit of detection and quantification demonstrate an increased sensitivity of the sensor to determine simultaneously FA and AC but also the feasibility of the voltammetric method used. The LOD and LOQ values obtained in the present study are like those presented in another research [132–134].

7.1.4. Interference studies

To investigate the simultaneous determination of different interference CA and FA were added sequentially, in solution, vanillic acid, gallic acid and quercetin. Initially, concentrations of 5×10^{-4} M were added in turn, for each interference. Signal changes were imperceptible. Then higher concentrations of each compound (10^{-3} M) were added to test the tolerance limit. Quercetin had the most influence over the peaks, due to the similar chemical structure of CA and FA. The results are presented in Table 7.3.

Table 7.3. Interference of some chemically related species on the simultaneous determination of CA and FA (10^{-3} M).

Interfering compound	Concentration ratio	Recovery / %	Concentration ratio	Recovery / %
Quercetin	1:0.5	100 ± 4.6	1:1	96 ± 2.1
Vanilic acid	1:0.5	102 ± 1.9	1:1	98 ± 3.1
Gallic acid	1:0.5	102 ± 4.1	1:1	99 ± 3.5

7.1.5. Stability and repeatability of the sensor

To investigate the repeatability of the sensor, the voltammetric measurements were performed in a 10^{-3} M CA and FA solution (of both compounds), with the same sensor 10 times. The relative standard deviation (RSD) of the measurements was 4.5%. Between measurements, CNF / C-SPE was rinsed with 0.1 M PBS pH 7.0. Therefore, CNF / C-SPE can be used repeatedly.

The stability of the sensor was verified by monitoring the voltammetric response in a solution with equal concentration of AC and FA (10^{-3} M) at regular intervals (1 day) for a period of one month. During this time the sensor was stored at 4°C in a refrigerator. The electrochemical response was maintained at 90%.

7.1.6. Simultaneous determination of CA and FA in the Eye Blend product

To confirm the feasibility of the method, the Eye Blend product was chosen for the detection of CA and FA from its composition, using CNF / SPE. In Figure 7.8. the first 5 CV scans are presented, recorded with CNF / SPE immersed in the solution obtained by dissolving Eye Blend capsules in PBS 10^{-1} M, pH 7.0.

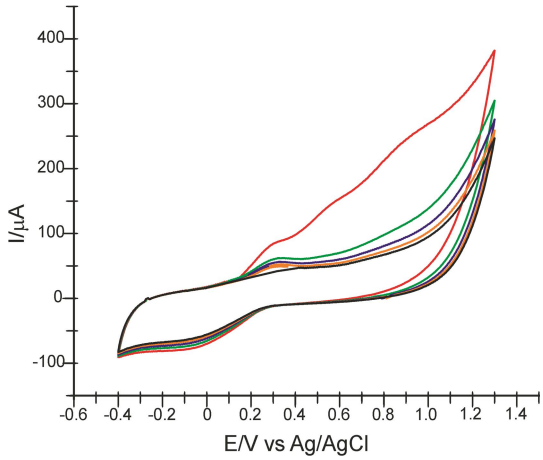

Figure 7.8. Cyclic voltammograms recorded with CNF / C-SPE immersed in Eye Blend solution (1 cps / 50 mL PBS 0.1M, pH 7.0): first scan-line red, second scan-line green, third scan-line blue, fourth scan-line orange, fifth scan-line black

It is noted the appearance of the first anodic peak of the CV scan reflecting the presence of ferulic acid. On the following scan, the second and third peak intensity decrease because of the deposition process of AF on the electrode surface. The decrease in intensity of the two peaks could also be due to the presence in the composition of other electroactive

compounds, which inhibit the activity of FA. The quantification of FA and CA will be done by means of the cathodic peak. Cathodic peak potential for the presence of CA is close to the FA, so that it is difficult to determine the exact concentration of the compounds. However, the amounts of FA and CA were estimated, as can be seen in Table 7.3.

Table 7.4. Quantitative data for the determination of CA and FA in the Eye Blend product using CNF / C-SPE

Phenolic compound detected	Voltammetric method	
	% c	mg/caps
CA	6.021	21.076
FA	7.516	26.306

7.1.1. Determination of the antioxidant capacity of CA and FA by the DPPH method

The DPPH method is based on the reaction with electron donors or hydrogen radicals (H *) that produce antioxidant compounds. The reduction of DPPH is directly proportional to the amount of antioxidant present in the analysed sample [135].

It was observed that the reduction of DPPH was dependent on the concentration of the phenolic compound in the sample. When equal concentrations of FA and CA (50 μL) were added to the DPPH solution, the reagent reduction process was more intense, resulting in a lower absorbance value. This demonstrates that the existence of both phenolic compounds in the probe (although in a lower concentration than that in which they are taken separately), favours a greater antioxidant effect.

Radical scavenging activity was expressed as a percentage and was calculated using the following formula:

$$\%C = (A_{control}-A_{probe}) / A_{probe}X100$$

Table 7.5. Determination of the antioxidant activity of CA and FA compounds

Probe volume	3.22×10^{-5} M CA	6.25×10^{-5} M CA	3.22×10^{-5} M FA	6.25×10^{-5} M FA	1.63×10^{-5} M CA + 1.63×10^{-5} M FA	100 μL Eye Blend
% c antiox activity	0.971922	1.523395	0.873362	1.898188	9.67391304	14.55767

It can be stated that CA and FA are present in the composition of the Eye Blend product in close quantities. The studied method can be considered a cheap and easy to handle tool that could be applied to various other pharmaceuticals.

7.2. Conclusions

The study aimed at the simultaneous determination of two phenolic compounds, caffeic acid and ferulic acid, from a phyto-homeopathic formula. In the experimental tests was used a screen-printed electrode modified with carbon nanofibers. The electrochemical method used was cyclic voltammetry. The electrochemical behaviour of the CNF / C-SPE has been analysed in the previous section.

The sensor showed a good sensitivity for the detection of both analytes in the test solution. Quantitative FA and CA were determined from the Eye Blend product with CNF / C-SPE by cyclic voltammetry. Also, the influence of other species on the electrochemical signal was reduced, the sensor having favourable specificity. Subsequently, the antioxidant activity of the compounds was determined by the DPPH method, thus demonstrating the antioxidant, synergistic effect of the two phenolic compounds in the Eye Blend product.

Therefore, both the sensor and the voltammetric method used have been proved to be suitable for the simultaneous determination of the two phenolic compounds in a product with a complex composition. It can be stated that this detection method is sensitive, accurate, easy to apply and could also be used for simultaneous determinations of other phenolic compounds.

CHAPTER 8. Development of new electrochemical biosensor based on carbon nanofibers, cobalt phthalocyanine and laccase for the detection of p-coumaric acid in phytopreparations

Introduction

The aim of this work is to evaluate the electrochemical behaviour of a new carbon-based biosensor modified with nanofibers, cobalt phthalocyanine and laccase (CNF-Copc-Lac-SPE) on the voltammetric detection of PCA using various techniques. Also, the electroanalytical method will be validated for the quantification of PCA in different phytopreparations products using the FTIR spectrometric method.

8.1. Materials and methods

8.1.1. Preparation of the CNF-CoPc / SPE sensor

For the preparation of the modified CNF-CoPc / SPE sensor: on the surface of the screen-printed electrode modified with carbon nanofibers was added, by drop-and-dry technique, a quantity of 10 μL solution of 10^{-5} M cobalt phthalocyanine in chloroform, sequentially, with breaks for drying. Drying was performed at room temperature for 30 minutes. The addition of cobalt phthalocyanine solution was performed using an Eppendorf micropipette.

8.1.2. Preparation of the CNF-CoPc-Lac/SPE biosensor

For preparation of the biosensor was used as support CNF-CoPc / SPE. By the casting technique was added a volume of 10 μL, sequentially, in two stages, with a drying break between the two additions, of 3 hours. Crosslinking of the enzyme was performed by positioning the sensor over a container with 2 mL of 2% glutaraldehyde for 1 minute.,

Glutaraldehyde vapours ensured the immobilization of the laccase on the electrode surface. Biosensors were stored at 4°C until use, maximum 72 hours [98]. Figure 8.2. shows the preparation process of the biosensor a) and the enzymatic oxidation mechanism of PCA in the presence of laccase b).

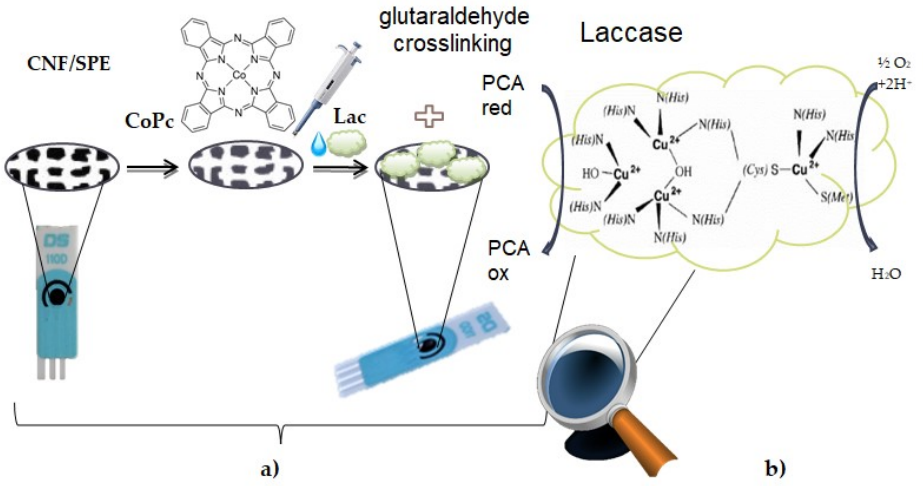

Figure 8.2. a) The process of preparing the laccase-based biosensor based on a screen-printed electrode based on carbon nanofibers modified with cobalt phthalocyanine. b) the mechanism of enzymatic oxidation of PCA in the presence of laccase

8.2. Results and discussions

8.2.1. Preliminary studies for electrode characterization

To observe the changes made to the commercial sensor based on carbon nanofibers, the active surface of the two working electrodes was analysed with the FTIR spectrophotometric method.

In the FTIR spectra for CNF-Co-Pc / SPE, respectively CNF-Co / Pc-Lac / SPE, the differences are obvious, both as number of peaks and as background noise. Several peaks can be observed representing the presence of laccase, in the wavelength range 1460-1620 cm^{-1} [136].

In the preliminary analyses, the electrochemical behaviour of CNF / SPE, CNF-CoPc / SPE and CNF-CoPc-Lac / SPE in 10^{-1} M phosphate buffer solutions with different pH values (3.0, 4.0, 5.0, 6.0) was evaluated. According to previous studies, stable signal was obtained in the potential range of -0.4 to + 1.3V [3]. Therefore, the potential field has been used to study the electrochemical behaviour of the electrodes immersed in 10^{-1} M PBS (pH = 3.0, 4.0, 5.0, 6.0) at a scan speed of 0.1 $V \times s^{-1}$.

In the cyclic voltammograms obtained with CNF / SPE no peaks were observed in the studied range of potential (results not shown), demonstrating that the active surface of the electrode does not show contamination and carbon nanofibers have high purity.

When the CNF-CoPc / SPE sensor was immersed in phosphate buffer solution at different pH values, the CV showed two peaks, one anodic, of low intensity and one cathodic, more obvious. The cathodic peak current increased with increasing pH. At pH = 5.0, the anodic peak appears at 0.67 V and the cathodic peak at -0.21 V. The peaks are related to the oxidation-reduction process of CoPc on the surface of the modified electrode and are consistent with the results obtained in other studies [137].

Prior was investigated the electrochemical reduction of the laccase on the biosensor surface in PBS solution with pH values between 3.0 and 6.0, and the CV responses indicated that the I_{pc} increases proportionally to the increase in pH, up to pH = 5.0. At pH = 6.0, the I_{pc} I had a considerable decrease. Moreover, increasing the pH to 5.0 resulted in a linear shift of the cathodic peak potential to more negative values. The regression equation is Ep = -0.0362 pH + 0.0567.

8.2.2. The voltammetric response of the electrodes in the p-coumaric acid solution

According to preliminary studies but also to the literature, it was found that the optimal pH value for the detection of phenolic compounds is 5.0 [138].The peaks obtained at this pH value are more obvious and well defined [139][140,141]. A higher peak intensity indicates that the immobilization step did not adversely affect the enzyme activity. In addition, a lower pH value could contribute to the faster degradation of the enzyme. Therefore, in the following experimental analyses, the 10^{-1} M PBS solution (pH = 5.0) was used as the support electrolyte.

Working electrodes were used to record cyclic voltammograms, using a 10^{-3} M p-coumaric acid solution (PBS 10^{-1} M pH = 5.0). The scan speed used was 0.1 V/s. Cyclic voltammograms look slightly different depending on the changes made to the working electrode. At the first voltammetric scan, a well-marked, irreversible anodic peak appears in each situation, which is associated with the oxidation of the hydroxyl group on the aromatic ring of the molecule and the formation of phenoxy radicals, which can subsequently dimerize or polymerize [142]. In the case of the biosensor, the anodic peak occurs at a potential of 0.904 V.

At successive scans, the oxidation product of p-coumaric acid is deposited on the electrode surface forming a polymeric film, thus explaining the appearance of another reversible oxidation peak, at a lower potential than that of p-coumaric acid [143]. The increase in the intensity of the reversible oxidation peak is explained by the increase in thickness of the polymeric film covering the electrode surface and prevents the diffusion of p-coumaric acid and its oxidation on the electrode surface [143].

When using CNF-CoPc-Lac / SPE, the maximum potential for the oxidation product occurs at E_{pa} = 0.537 V, and the reduction peak has an E_{pc} potential = 0.011 V. Thus, the quasi-reversibility of the oxidation process is confirmed. These values are like those found in other works studying the oxidation-reduction of p-coumaric acid [143]. When the oxidation product is

adsorbed on the electrode surface, the p-coumaric acid oxidation peak decreases in intensity, while the oxidation product peak increases in intensity on successive scans (Figure 8.8.).

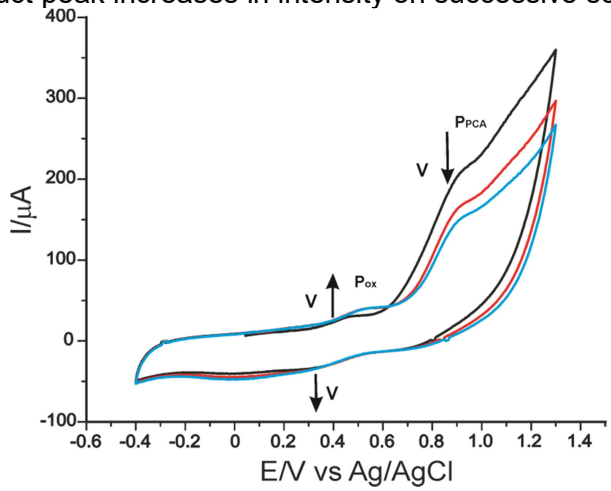

Figure 8.8. CNF-CoPc-Lac / SPE cyclic voltammogram immersed in 10^{-3} M p-coumaric acid solution (10^{-1} M PBS electrolyte, pH = 5.0): first scan (black line), second scan (red line) and the third scan (blue line).

The peak of the oxidation product has a lower oxidation potential than that of p-coumaric acid due to the formation of organic polymers by oxidation [143].

CNF-CoPc-Lac / SPE is distinguished by a low value of the cathodic peak potential, which means that the reduction process requires a lower activation energy and is influenced by the presence of laccase [52][118][120]. Also, the low value of E_{pc} suggests a rapid process of electron transfer in the redox process of PCA at the active surface of the biosensor [122].

Therefore, CNF-CoPc-Lac / SPE has a better selectivity compared to the other two sensors for the detection of p-coumaric acid, thus confirming the biocatalytic activity of the laccase immobilized on the surface of the biosensor. The values of the parameters $E_{1/2}$ and I_{pc}/I_{pox} prove that the biosensor has a higher sensitivity.

In addition, the cathodic peak is visibly more intense, which is why subsequent calculations will relate to its changes. In the case of CNF-CoPc-Lac / SPE, the signal was more stable and the background noise lower. The PCA reduction process has been studied and has been shown to occur at a low potential and is due to the pre-protonated conjugate double bond [144]. Since the p-coumaric acid molecule contains an oxidizable phenolic group on the aromatic ring, it can be stated that this compound can be determined voltammetrically.

Laccase can catalyse the oxidation process of p-coumaric acid. Peaks obtained at a scan speed of 0.1 V/s have low intensities and are less visible due to the influence of capacitive current. At higher scanning speeds, farad currents are higher, and peaks are better defined [145].

The same electrodes were used to record square wave voltammograms in the 10^{-3} M p-coumaric acid solution (PBS electrolyte 10^{-1} M pH = 5.0). The studied potential range was between -0.4 and + 1.3V, the pulse height, 0.09 V, an increase of the pulse potential of 7 mV and a frequency of 15 Hz. This technique showed similar results to cyclic voltammetry. With all three electrodes, two oxidation peaks were highlighted. In each situation there are better defined peaks and lower background current.

With the help of SWV, the reversibility of the peak related to the oxidation product (I_{pox}) and the irreversibility of the second peak (I_{PCA}) are highlighted. In the case of CNF-CoPc-Lac / SPE the first anodic peak was observed at 0.392 V, and the second at 0.885 V.

There is a much smaller difference between the intensity of the first anodic peak and the second, which confirms that the PCA is absorbed on the sensor surface, interacting with the immobilized laccase.

In the next step, the electrochemical behaviour of the three electrodes in 10^{-3} M PCA solution was studied (the supporting electrolyte was 10^{-1} M PBS at pH 5.0), applying scanning speeds increasing in the range 0.1-1.0 V/s. Remarkable differences are observed between the intensities of oxidation and reduction currents and the measured potentials. From the second applied scanning speed, the peaks increase progressively with increasing scanning speed.

Given that the presence of the enzyme mainly influences the cathodic peak, the dependence of I_{pc} will be studied depending on the scanning speed.

It was determined that there is a linear dependence between the cathodic peak currents and the scanning speed for all three electrodes (Table 8.2.). This fact indicates that the process that takes place on the surface of the electrodes is controlled by the adsorption of the electroactive species [143].

Given the equation of linear dependence between the cathodic peak current and the scanning speed, the coverage of the electrode surface with the electroactive species (Γ) was calculated using Laviron's equation, and the results are presented in Table 8.2. [146].

Comparing the results obtained with the three electrodes, it can be stated that, in all cases, the reduction process is controlled by PCA adsorption on the active surface, which is faster and more obvious in the case of the biosensor.

Table 8.2. Linear dependence equations (Ipc vs. v), R2 and Γ for the three electrodes used in the analysis

Electrodes	Linear equation	R^2	Γ (mol×cm^{-2})
CNF/SPE	$I_{pc} = -197.46\times10^{-6}\ v - 7.5975\times10^{-6}$	0.996	3.84×10^{-10}
CNF-CoPc/SPE	$I_{pc} = -229.75\times10^{-6}\ v - 11.029\times10^{-6}$	0.999	4.46×10^{-10}
CNF-CoPc-Lac/SPE	$I_{pc} = -310.01\times10^{-6}\ v - 41.585\times10^{-6}$	0.982	6.02×10^{-10}

From these results it can be appreciated that CNF-CoPc-Lac / SPE has superior electroanalytical properties for PCA detection. In addition, the presence of laccase ensures the selectivity of the biosensor and can be used in the analysis of complex samples.

Immobilization of the laccase together with carbon nanofibers and cobalt phthalocyanine leads to better bioselectivity and conductivity, these nanomaterials having a synergistic effect in biodetection [147]. Since CNF-CoPc-Lac / SPE proved superior performance on sensitivity and selectivity, it will be used in subsequent quantitative analysis.

8.2.3. Calibration curve

To achieve the calibration curve, cyclic voltammograms of p-coumaric acid were recorded, at the successive addition of variable quantities, between 5 μL and 30 μL, of 10^{-3} M p-coumaric acid stock solution in 50 mL PBS pH = 5 followed by stirring. After homogenization of the analysed solution, cyclic voltammograms were recorded. The concentration range studied was 0.1 μM - 202.5 μM. The cathodic peak current increases with increasing PCA concentration. The anodic peak current was linear in the range of 0.4-6.4 μM.

Using the linear regression equation, LOD (3σ/m, where σ was the standard deviation and m was the slope of the calibration curve) and LOQ (10σ/m) [148] were calculated and the values are shown in Table 8.3.

Table 8.3. Equation of linear dependence between I_{pc} and c, R^2, LOD and LOQ for CNF-CoPc / SPE and CNF-CoPc-Lac / SPE

Electrodes	Linear equation	R^2	LOD (M)	LOQ (M)
CNF-CoPc/SPE	y = -0.0114x – 0.4352	0.9785	9.29×10^{-7}	3.1×10^{-6}
CNF-CoPc-Lac/SPE	y = -0.0247x – 0.4188	0.9714	4.83×10^{-7}	1.61×10^{-6}

The biosensor has superior performance to the sensor, due to the presence of the enzyme that gives it selectivity and sensitivity and promotes the interaction with p-coumaric acid. A calibration curve for the same concentration range (0.1 μM - 202.5 μM) of p-coumaric acid was also performed by chronoamperometry (Figure 8.14.) for CNF-CoPc-Lac / SPE, at a potential kept constant at -0.2 V. The LOD and LOQ values obtained were 1.63×10^{-7} M, respectively 5.42×10^{-7} M, close to those obtained by CV.

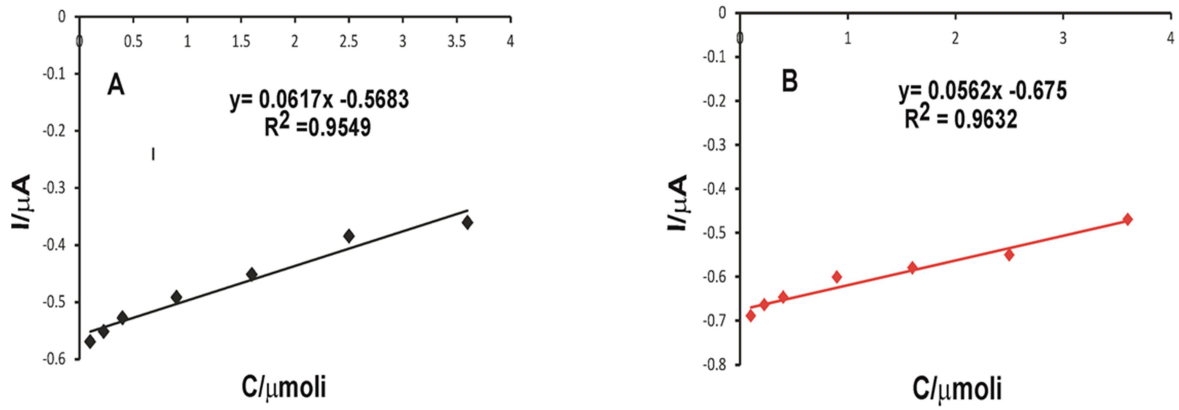

Figure 8.14. Linear adjustment in the range 0.1-3.6 μM for A) CNF-CoPc-Lac / SPE and B) CNF-CoPc / SPE.

The low values of the limits of detection and quantification are in accordance with the values obtained by other types of sensors or biosensors capable of determining a hydroxycinnamic acid. The biosensor has a high sensitivity, with a favourable difference in the case of the biosensor, due to the immobilization of the laccase, an enzyme that also gives it selectivity.

Voltamperometric methods have proven to be feasible for the analysis of p-coumaric acid in various real samples, such as phytopreparations, useful in maintaining health or adjuvants in treating diseases. For the quantitative determination of p-coumaric acid in the selected phytopreparations, the new enzyme sensor developed in this study can be used successfully.

8.2.4. Stability, reproducibility and repeatability of the biosensor

The stability of the biosensor has been studied and it has been found that it can be used for more than 30 measurements by cyclic voltammetry in PCA-containing solutions. To verify the reproducibility of manufacturing methods, the response of two identically prepared biosensors in PCA solutions of the same concentration was studied . No differences of more than 2% were observed between the two biosensors. Also, the variation of the biosensor response to the determination of PCA in solution to calculate the concentration, to removal from solution, rinsing and repetition of the cyclic voltammogram did not exceed 3%.

8.2.5. Interference studies

For interference studies the biosensor behaviour was evaluated, in addition to PCA-related chemical compounds, which are often found in phytopreparations, for example gallic acid, ascorbic acid, vanillic acid, ferulic acid. The PCA solution had a concentration of 50 μM, adding the same concentration of interferences.

The determination of PCA is not significantly influenced by interfering compounds. Peaks related to the other compounds are highlighted, but the anodic peaks and the cathodic peak of the PCA are not affected. From these results, it can be concluded that the CNF-CoPc-Lac / SPE sensor has a good accuracy and selectivity for the determination of PCA from real samples.

8.2.6. Determination of PCA in phytopreparations

Phytopreparations selected for analysis have different presentation forms: solid cream, cream and tablets and a composition rich in antioxidants. The manufacturer does not specify an exact PCA concentration on the package leaflet, so the quantitative determination by the voltammetric method will be validated by a classical determination method. Well-established amounts of each product were used to obtain the solutions to be analysed (Ghindazine 1g, Thiazine 1g, Spirulina 0.75 g).

The intensity of the cathodic peak, corresponding to the potential -0.2 V, was used for quantification, in the case of each product. The results are included in Table 8.6.

Table 8.6. PCA concentrations in phytopreparations obtained by the voltammetric method, respectively the FTIR method

Phytopreparations	**FTIR method mg/g PCA**	**Voltammetric method mg/g PCA**
Spirulină	1.569	1.674
Ghindazin	0.644	0.783
Tuiazin	1.936	2.149

The FTIR method was used to validate the voltammetric method. For the spectrometric analysis, five standard samples with different concentrations of p-coumaric acid were prepared: 1, 2, 3, 4, 5 mg/g, with KBr. Samples from phytopreparations were analyzed without prior preparation. The experiments were analyzed in triplicate.

A calibration curve was performed according to the absorbance corresponding to the peak at 1238 cm^{-1}, a peak related to the elongation vibration of the phenolic C-O group [149]. The amounts of p-coumaric acid in the phytopreparations were calculated from the calibration equation.

The values of PCA concentrations in the real samples calculated with the spectrometric method are close to those obtained by the voltammetric method, the data being presented in Table 8.6. Thus it is certified the efficiency, sensitivity and selectivity of the enzyme sensor based on carbon nanofibers modified with cobalt phthalocyanine and laccase.

8.3. Conclusions

This study demonstrated the feasibility of developing modified sensors: the first with cobalt phthalocyanine and the second with cobalt phthalocyanine and laccase for the determination of p-coumaric acid in phytopreparations. From the obtained results we can conclude that cobalt phthalocyanine favoured the activity of laccase, being also a mediator of electron transfer in the oxidation process of p-coumaric acid.

The voltammetric methods used in biodetection were cyclic voltammetry and square wave voltammetry. Cyclic voltammetry and chronoamperometry were used to study the electrochemical behaviour of the biosensor in the chosen concentration range.

The enzymatic biosensor has a high sensitivity and selectivity for the amperometric detection of hydroxycinnamic acid. The LOD and LOQ values obtained by CNF-CoPc-Lac / SPE are close to those obtained by other laccase-based biosensors for the detection of other phenolic compounds (Table 8.4.).

The PCA concentrations obtained by CNF-CoPc-Lac / SPE were close to those obtained by the FTIR spectrophotometric method.

In conclusion, the new biosensor developed based on cobalt phthalocyanine and laccase has multiple advantages such as: sensitivity, selectivity, feasibility, and low cost. CNF-CoPc-Lac / SPE can also be used in routine analyses to control the quality of nutraceutical, food or pharmaceutical products.7

GENERAL CONCLUSIONS. PERSONAL CONTRIBUTIONS. FUTURE RESEARCH PERSPECTIVES

GENERAL CONCLUSIONS

The thesis entitled "Development of new voltammetric sensors and biosensors for the determination of hydroxycinnamic acids" includes both a topical theoretical study, well documented and original contributions on the determination of hydroxycinnamic acids from various phytopreparations or cosmetics, using new sensors and electrochemical biosensors to obtain accurate results, at a relatively low cost ad in a short time.

The interest for this group of phenolic compounds comes from the fact that hydroxycinnamic acids (such as ferulic, caffeic, synaptic and p-coumaric acids) represent about a third of the phenolic compounds in people's diet. Food supplements are found in different forms of presentation, with an increasingly complex composition but with concentrations of biologically active compounds often unknown. Cosmetic formulations also offer a wide range of benefits for skin health and beauty, and the proportions between the active ingredients have proven to be of particular importance.

The most important biological activity of hydroxycinnamic acids is the antioxidant one due to their chemical structure, which is why they are increasingly identified in commercial products of various types. The mechanism of free radical scavenging is explained by the ability of hydroxycinnamic acids to clone a hydrogen atom or an electron, thus forming a stable phenoxyl radical. In the case of ortho-diphenols, it has been shown that the transfer of two electrons per molecule leads to the subsequent formation of an ortho-hydroxyphenoxyl radical and an ortho-quinone. These mechanisms can be explained in detail by electrochemical studies.

This information led to a study on voltammetric techniques used to characterize and quantify these compounds. The analytical advantages of voltammetric techniques include excellent sensitivity with good linearity in a wide range of concentration, short analysis time and the possibility of simultaneously determining compounds with similar structure. The differences between the main voltammetric techniques consist in the application of the potential and the measurement of the resulting current.

Subsequently, we studied carbon nanomaterials, such as graphene, graphene oxide, fullerene, carbon nanocons, diamond nanoparticles, carbon quantum particles, carbon nanofibers, carbon nanotubes, used to modify electrodes. Numerous advantages have been highlighted, such as a high surface-to-volume ratio and a large specific surface area, efficient interfacial adsorption, a higher electron transfer speed and good electrocatalytic properties. The incorporation of carbon nanomaterials can be done by dispersion in the solvent, the formation of a carbon paste and screen printing.

In experimental studies we chosed to use screen-printed sensors based on carbon nanomaterials with excellent properties.

Choosing an appropriate immobilization technique is essential for the developing of biosensors. One of the simplest methods of immobilizing the enzyme is by physical adsorption. Enzymes can also be immobilized by incorporation into three-dimensional matrices, such as an electropolymerized film or a carbon paste. Crosslinking is another very common approach to immobilization, which involves the use of a bifunctional compound, such as glutaraldehyde. Enzymes can also be immobilized by affinity bonds between an existing functional group on the support and a specific group present in the enzyme sequence.

All methods have multiple advantages, but also limitations. We can prevent some of the difficulties that may arise, choosing an immobilization method adapted to the enzyme that will be incorporated but also to the biomolecule to be detected. Also, by approaching a suitable immobilization technique we can avoid the instability or degradation of the enzyme. The immobilization process must be simple, reproducible, with a low cost and the shortest processing time.

All this information helped me to choose analyses of interest, working methods, type of sensors and nanomaterials used, optimization of experimental parameters, proper selection of

enzymes and immobilization techniques, tested products and finally the appropriate classical methods. to validate the results obtained.

PERSONAL CONTRIBUTIONS

For the experimental studies, three of the hydroxycinnamic acids (caffeic, ferulic and p-coumaric acid) were selected, these being among the most common phenolic compounds from plant sources.

In the first study, caffeic acid was accurately determined in food supplements with screen-printed carbon sensors modified with various nanomaterials, graphene, carbon nanofibers and multilayer carbon nanotubes, using cyclic voltammetry as a detection method. The characterization of the sensors by cyclic voltammetry in the reference solutions showed that carbon nanotubes or carbon nanofibers significantly improve the sensor response in terms of sensitivity and reversibility of electrochemical processes.

Screen-printed sensors were then used to study the electrochemical behaviour of caffeic acid in aqueous solution at pH 3.6. In all cases a reversible redox process was observed involving the transfer of two electrons and two protons. The role of nanomaterials in improving the electroanalytical performance of sensors has been highlighted. Calibration curves were performed for each sensor and detection limits (LOD) and quantification (LOQ) were calculated. Low values of LOD and LOQ, in the range of 10^{-7} to 10^{-9} M were obtained, which showed that the method is feasible for quantifying caffeic acid in real samples. Caffeic acid was determined quantitatively in three dietary supplements using the most sensitive sensor, namely the screen-printed sensor based on carbon nanofibers.

The Folin-Ciocalteu spectrophotometric method was used to validate the results obtained with the screen-printed sensor based on carbon nanofibers. The results obtained by using the voltammetric method were consistent with those obtained by using the spectrophotometric method, without statistically significant differences between the results obtained. Also, caffeic acid and other phenolic compounds in the composition of the tested food supplements were highlighted by the technique of high-performance chromatography (HPLC).

The second study refers to the electrochemical behaviour of modified screen-printed electrode types (SPE): a sensor based on carbon nanofibers (CNF / SPE), a sensor based on carbon nanofibers modified with gold nanoparticles (CNF-GNP / SPE) and a biosensor based on carbon nanofibers modified with gold nanoparticles and tyrosinase (CNF-GNP-Ty / SPE). To construct the biosensor, tyrosinase (Ty) was immobilized on the surface of the electrode modified with carbon nanofibers and gold nanoparticles (commercial sensor), by the drop-and-dry technique.

The electrochemical properties of the three electrodes were studied by cyclic voltammetry in electroactive solutions, and the potential, current and shape of the peaks were depending on the nature of the materials used to modify the electrodes. In the case of ferulic acid, a series of characteristic peaks were observed, the processes being more intense in the case of the biosensor. The biosensor has also shown greater sensitivity and selectivity due to the immobilization of tyrosinase, a specific enzyme for phenolic compounds. The calibration curve was performed using CNF-GNP-Ty / SPE in ferulic acid solutions of different concentrations in the range of 0.1–129.6 μM. This new biosensor allowed low values of detection and quantification limits, 2.89×10^{-9} mol·L^{-1} and 9.64×10^{-9} mol·L^{-1}, respectively, which shows that the electroanalytical method is feasible for the quantification of ferulic acid in real samples. Ferulic acid was determined quantitatively in three cosmetics using the CNF-GNP-Ty / SPE biosensor. The results obtained were validated by means of the spectrometric method in the infrared field, the differences between the values of ferulic acid concentrations obtained by the two methods being below 5%.

For the third experiment, simultaneously determination ferulic acid and caffeic acid from a phyto-homeopathic product (Eye Blend-Secom) was performed. The two compounds are mentioned in the manufacturer's specifications but without indicating the exact quantity. The aim of the study was to detect both compounds simultaneously and to quantify them from the phyto-homeopathic product, using a sensor characterized in another study, CNF / SPE. The stability and reproducibility of the CNF / SPE electrode proved to be effective, and the sensitivity was

high, both for AC (2.39×10^{-7} M) and for FA (2.33×10^{-7} M). Also, the antioxidant capacity of the compounds in the analysed product was determined, by the DPPH method. The electrochemical method was efficient and less expensive than other methods of analysis, therefore its use can be extended to detect these phenolic compounds in various pharmaceuticals.

In the last chapter of the experimental part, a new enzymatic biosensor based on laccase and cobalt phthalocyanine (CNF-CoPc-Lac / SPE) was developed to determine p-coumaric acid from three types of phyto-preparations with different presentation forms. The voltammetric techniques used in the preliminary analyses but also for determining the compound in the real samples were cyclic voltammetry and square wave voltammetry.

The modification of the sensors was performed by the casting and crosslinking technique, using glutaraldehyde as a crosslinking agent.

Preliminarily, the electrochemical reduction of the laccase on the biosensor surface in PBS solution with pH values between 3.0 and 6.0 was investigated, and the recorded voltammograms indicated that the cathodic current intensity increases in direct proportion to the pH increase, to pH = 5.0. At pH = 6.0, the I_{pc} had a considerable decrease. Moreover, increasing the pH to 5.0 resulted in a linear shift of the cathodic peak potential to more negative values, which means lower activation energy and better selectivity.

The calibration curve was performed for the p-coumaric acid concentration range of 0.1–202.5 μM, using cyclic voltammetry and chronoamperometry. With the two modified electrodes we obtained optimal results, in the linearity range 0.4-6.4 μM, CNF-CoPc-Lac / SPE being distinguished by lower LOD and LOQ values, 4.83×10^{-7} M respectively 1.61×10^{-6} M. P-coumaric acid was successfully determined from three phytopreparations with complex composition. The results obtained by the voltammetric method were comparable to those obtained by the FTIR method. The amount of p-coumaric acid determined with CNF-CoPc-Lac / SPE was closer to that obtained by the spectrometric method.

From all the experiments undertaken it can be concluded that:

- Carbon nanomaterials and metallic or semiconductor nanoparticles can improve the performance of enzymatic sensors or biosensors, providing outstanding electrochemical and mechanical properties
- Electrochemical parameters such as potential range or scan speed must be optimized so that the signal is stable, and the background noise is reduced
- The pH of the solutions has a great influence on the stability and responses of biosensors
- The active surface area of the sensor / biosensor, the diffusion coefficient or the concentration of the adsorbed active species are important parameters in the evaluation of the redox mechanisms that occur at the electrode surface
- Tyrosinase and laccase can oxidize hydroxycinnamic acids to quinone compounds, which are electroactive
- The process of recognizing substrates is influenced by the activity of enzymes
- The drop-and-dry technique followed by crosslinking with glutaraldehyde proved to be suitable for the development of the biosensor, the biocatalytic activity of the enzyme being unchanged after immobilization
- Cobalt phthalocyanine is an electron transfer mediator compound, improving the response of the laccase-based biosensor.
- The sensors and biosensors developed in these studies show values of low detection and quantification limits, extended linearity intervals, with similar performances to the sensors reported in the literature
- The results of quantitative determinations of hydroxycinnamic acids from real samples were validated by classical detection methods such as Fourier transform infrared spectroscopy, high performance liquid chromatography and the method of determining antioxidant activity.
- The sensors and biosensors developed in these studies have proven to be accurate and feasible, so they can be used in routine analysis, in quality control of cosmetics, pharmaceuticals, food supplements and other types of samples.

FUTURE RESEARCH PERSPECTIVES

Studies conducted in this paper have shown that enzymatic biosensors are precise, selective, and effective tools to determine hydroxycinnamic acids in various commercial products. Improving the immobilization technique, using new nanomaterials or polymeric compounds as mediators, and optimizing the working parameters could lead to the design of portable biosensor systems for a routine, rapid, relatively low-cost testing of food supplements or phytopreparations. The characteristics of carbon nanomaterials could be exploited to incorporate other enzymes capable of keeping their biocatalytic activity unchanged after immobilization, to develop sensitive devices, with extended applicability in various fields of research.

Of particular interest may be the development of portable and miniaturized sensors for the simultaneous detection of hydroxycinnamic acids in food samples, for example vegetable oils because these compounds have beneficial effects on the human body and at the same time can be used as biomarkers of authenticity.

Another innovative approach could be the development and use of sensitive devices that, installed, or associated with a smartphone, can form independent platforms for the detection of various analytics. These tools could transmit signals through the built-in USB port, wireless or other integrated components, and they could also display and interpret the results through a specialized mobile application. Open-source mobile applications could be a starting point for routine testing of food supplements and non-standardized pharmaceuticals. The proposed research topic can be a step towards the realization of such a sensitive device that could be used in everyday life in the food, pharmaceutical or cosmetics industry.

List of published studies

Bounegru Alexandra Virginia- https://orcid.org/0000-0002-6005-492X

Book chapter

Apetrei, C.; **Bounegru, A.V.** Electronic Noses and Traceability of Foods. In Comprehensive Foodomics; Elsevier, 2021; pp. 290–307 ISBN 978-0-12-816396-2.

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- **Alexandra Virginia Mereşescu (Bounegru)**, Constantin Apetrei. Electrochemical Determination Of Ferulic Acid In Cosmetics Using Screen-Printed Carbon Nanofiber Electrodes Modified With Gold Nanoparticles. New Trends on Sensing- Monitoring-Telediagnosis for Life Sciences NT SMT-LS 2020 Brasov, Romania, July 3-4, 2020.
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- Constantin Apetrei,*, **Alexandra Virginia Bounegru**, Irina Georgiana Munteanu, Irina Mirela Apetrei Development of a sensitive method for the voltammetric detection of phenolic compounds in extra virgin olive oils. CSAC2021: 1st International Electronic Conference on Chemical Sensors and Analytical Chemistry. 01-15.07.2021
- **Alexandra Virginia Bounegru**, Constantin Apetrei. Development of novel biosensor for the detection of p-coumaric acid in phenolic extracts from virgin olive oils. 31st Anniversary World Congress on Biosensors. 26-29 July 2021.

Awards obtained

- First prize –Poster sesion 2019. Alexandra Virginia Mereşescu (Bounegru), Constantin Apetrei. Poster: ”Development of Screen-Printed Sensors Based on Carbonaceous Nanomaterials”. SCDS-UDJG 2019, The Seventh Edition, Galaţi, 13th-14th of June 2019.
- Bronze medal, 2019. Alexandra Virginia Mereşescu (Bounegru), Constantin Apetrei. Poster: Development of screen-printed sensors based on carbonaceous nanomaterials for the determination of caffeic acid. UGALINVENT, Research and Innovation Salon, Ediţia a IV-a, 2019.
- Mention- Poster sesion 2020. Alexandra Virginia Mereşescu (Bounegru), Constantin Apetrei. Poster: Enzyme Sensor Based on Carbon Nanofibers Modified with Gold Nanoparticle and Tyrosinase Used for Ferulic Acid Detection in Cosmetics. SCDS-UDJG 2020, Galați, 18th and 19th of June 2020.
- Second prize for the article “Voltamperometric Sensors and Biosensors Based on Carbon Nanomaterials Used for Detecting Caffeic Acid—A Review”, published inInternational Journal of Molecular Sciences at the competition for AWARDING THE RESULTS OF THE RESEARCH OF THE DOCTORAL STUDENTS FROM IOSUD-UDJG FOR THE YEAR 2020.
- Third prize- Poster sesion 2021. Alexandra Virginia Mereşescu (Bounegru), Constantin Apetrei. Poster: Enzyme sensors based on carbonaceous nanomaterials modified with cobalt phtalocyanine and lacasse used for p-coumaric acid detection in pharmaceuticals products. SCDS-UDJG 2021, Galați, 10th and 11th of June 2021.

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