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

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DOCTORAL THESIS
SUMMARY

THE DEVELOPMENT OF NEW ELECTROANALYTICAL METHODS FOR THE DETERMINATION OF BIOACTIVE COMPOUNDS WITH ANTIOXIDANT PROPERTIES

PhD Student,

Irina – Georgiana BULGARU (MUNTEANU)

Scientific guide,

Prof. univ. dr. chim. habil. Constantin APETREI

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Phd student Irina - Georgiana Bulgaru (Munteanu)

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I dedicate this thesis to my children...

CONTENT

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INTRODUCTION

In recent years, the use of antioxidants has increased considerably among the population, due both to the interest of the pharmaceutical industry in these compounds and to increased medical recommendations. Most life-giving biochemical reactions are associated with the production of free radicals, which in turn promote oxidative stress and contribute to body damage. Complex biochemical pathways in the human body are responsible for combating oxidative stress by ensuring an adequate level of balance between pro-oxidants (free radicals) and antioxidants. Epidemiological data suggested an inverse correlation between the consumption of fruits and vegetables, naturally rich in antioxidants, and the incidence of certain diseases (cardiovascular disorders, metabolic diseases, cancer) [1].

Recent advances in medicine and nutrition are changing the traditional approach of healthcare toward personalized medicine, which prioritizes disease prevention and health promotion, in particular through lifestyle and diet and nutrition-based approaches [2]. In this context, plant-derived antioxidants such as flavonoids, vitamins, hormones, phenolic acids and esters are considered bioactive dietary compounds capable of reducing oxidative stress and have been associated with numerous health benefits [3]. Antioxidants play an important role in maintaining optimal balance in the body [4], which has led to the analysis of these substances or antioxidant activity in various foods and beverages becoming a booming field of research [5]. Measuring the antioxidant activity of food and biological samples is therefore not only essential for ensuring the quality of functional foods but, more importantly, for the effectiveness of food antioxidants in the protection and treatment of diseases related to oxidative stress.

Of the multitude of secondary metabolites present in plants, phenolic compounds play a fundamental role against oxidative stress, representing one of the most synthesized and studied classes [6]. They are considered genuine antioxidants and are associated with benefits in reducing the risk of chronic diseases, as demonstrated by numerous reviews in the literature on their biological activity [7], [8].

Taking these considerations into account, it is considered necessary to develop user-friendly devices for monitoring the quality of nutraceutical products with declared content in such bioactive compounds or foods with an antioxidant role. Thus, in this paper, four compounds of major importance and with remarkable antioxidant properties were studied, namely three derivatives of caffeic acid, namely chlorogenic acid, rosmarinic acid and verbascoside, and a flavonic derivative, respectively catechin.

The studies carried out in the direction of these four compounds bring originality to the present research work, being detailed both in the theoretical part, respectively in the advanced documentation in the specialized literature, but especially in the second part of the thesis, respectively the experimental part. Thus, a series of promising and versatile electrochemical devices were developed and characterized, being subsequently used for the determination and quantification of the above-mentioned bioactive compounds, which are found in various nutraceutical formulations, in various forms of presentation, in cosmetics and also in other products. in foods represented by extra virgin olive oils. The development of such devices, namely chemically modified sensors and enzyme biosensors, was based on the use of state-of-the-art materials, including screen-printed carbon electrodes, electroactive mediators, enzymes and peptides, which showed excellent properties, contributing to the improvement of the sensitivity of newly created devices. In this paper, we studied all the stages included in the

manufacturing process of these sensors and biosensors, from preparation, modification, optimization of parameters, analysis proper and to validation of the results obtained at laboratory level both on real samples and by using other known methods of determination. Moreover, through these devices, it was possible to determine the antioxidant profile of the studied compounds taking into account the electrochemical parameters obtained from voltametric determinations, thus outlining a series of indications about the presumed antioxidant activity of each analyzed sample.

Techniques used to characterize newly developed devices have provided a variety of perspectives to understand and describe their properties. Thus, the methods used were also CV, SWV, DPV, SEM, HPLC, FT-IR, UV-viz spectroscopy. In addition to their application in fundamental studies of oxidation and reduction processes to discover reaction mechanisms, these techniques have also been used in studying the kinetics and thermodynamics of ion and electron transfer processes [9].

Taking all this into account, this doctoral thesis entitled 'Development of new electroanalytical methods for the determination of bioactive compounds with antioxidant properties' has achieved its purpose and objectives to develop sensitive and selective electrochemical devices, used for the determination and quantification of bioactive compounds of major importance for combating diseases associated with oxidative stress, through the antioxidant activity they manifest in the human body.

Keywords: chlorogenic acid, catechin, rosmarinic acid, verbascoside, sensor, biosensor, cyclic voltammetry, differential-pulse voltammetry, laccase, tyrosinase, manganese phthalocyanine, peptide, antioxidant activity.

LIST OF ABBREVIATIONS

AAPH - (2,2′-azobis(2-amidinopropane) dihydrochloride
ABS - acetate buffer solution
ABTS – 2,2 azinobis 3-ethylbenzothiazoline -6 – sulfonate
AFM - atomic force microscopy
BHA - butylhydroxyanisole
BHT - butylhydroxytoluene
BSA - bovine serum albumin
CAT - catalase
CGA – chlorogenic acid
CNT – carbon nanotubes
CPE - carbon paste electrode
CUPRAC - cupric reducing antioxidant capacity assay
CV – cyclic voltammetry
DMPD - N,N-dimethyl-p-phenylenediamine dihydrochloride
DPPH – 2,2-diphenyl-1-picrylhydrazyl
DPV - pulse-differential voltammetry
EDTA - ethylenediaminotetraacetic acid
EI - electrochemical index
EIS - electrochemical impedance spectroscopy
EPR - electronic paramagnetic resonance
ESI - electrospray ionization
Epa - anodic peak potential
Epc - cathodic peak potential
EVOO - extra virgin olive oil
FRAP - ferric reducing antioxidant power assay
FT-IR - Fourier-transform infrared spectroscopy
GA - glutaraldehyde
GC - gas chromatography
GCE – glassy carbon electrode
GNP – gold nanoparticles
GPH - graphen
GO – graphen oxide
GSH-Px - glutathione peroxidase
HORAC - hydroxyl radical antioxidant capacity
HPLC - high performance liquid chromatography
HSA - human serum albumin
Ipa - anodic peak intensity
Ipc - cathodic peak intensity
IR - infrared
Lac - laccase
LOD – limit of detection
LOQ – limit of quantification

MnPc - manganese phthalocyanin
MS - mass spectrometry
MWCNTs - multi-walled carbon nanotubes
OG – octyl gallate
ORAC - oxygen radical absorbance capacity
PBS - phosphate buffer solution
PCET - electron transfer coupled with proton transfer
PG - propyl gallate
Ppy - polypyrol
PVA - polyvinyl alcohol
ROS - reactive oxygen species
Sa - the area under the anodic peak
SEM - scanning electron microscopy
SPCEs - carbon screen-printed electrodes
SWV - square wave voltammetry
TAC - total antioxidant capacity
TEM - transmission electron microscopy
TOSC - total neutralization capacity of oxi-radicals
TPC - total polyphenol content
TPTZ - tripyridyltriazine
TRAP – total reactive antioxidant potential
Tyr - tyrosinase
UV - ultraviolet
Vis - visible

PART I. THE CURRENT STATE OF ART

CHAPTER I. GENERAL NOTIONS ABOUT BIOACTIVE COMPOUNDS WITH ANTIOXIDANT PROPERTIES

I.1. Spread and biological activity

Oxidative stress is a relatively new concept that has been widely involved in biomedical sciences for the past 20 years It significantly participates in the physiology of very widespread diseases such as diabetes, hypertension, preeclampsia, atherosclerosis, acute renal failure, Alzheimer's and Parkinson's disease. The metabolism of oxygen by cells generates potentially harmful ROS. Under normal conditions, the speed and extent of the formation of oxidants is balanced by the rate of their elimination. However, a negative imbalance between pro-oxidants and antioxidants leads to oxidative stress. Increased levels of ROS in the cell have a substantial impact, either leading to defective cell function, aging, or disease [10].

Numerous evidence suggests that antioxidants play an essential role in the maintenance of human health, in the prevention and treatment of these diseases due to their ability to reduce oxidative stress. The determination of antioxidant activity of food and biological samples is therefore not only essential for ensuring the quality of functional foods but, more importantly, for the effectiveness of food antioxidants in the protection and treatment of diseases related to oxidative stress.

Antioxidants are substances that, when present in food or in the body at very low concentrations, delay, control or prevent oxidative processes that lead to deterioration of food quality or the initiation and propagation of degenerative diseases in the body. These substances exert their inhibitory effect against oxidation processes through various mechanisms and with various activities [11].

I.2. Classification of antioxidants

According to the mechanism of action, antioxidants can be classified into primary and secondary antioxidants. Primary antioxidants inhibit the chain reaction of oxidation, acting as hydrogen donors or free radical accepters and generating more stable radicals. In this category are: Phenolic acids, flavonoids, tannins, carotenoids, stilbenes, coumarins and lignans. Secondary antioxidants are phenolic compounds that perform the function of capturing free radicals and stopping chain reactions. Compounds include: BHA, BHT, PG and OG [12].

I.3. Phenolic compounds – chemical structures and their role in the body

Phenolic compounds are commonly metabolites in various sources, especially plants, and are of major importance to human health due to their antioxidant activity, along with anti-inflammatory, antimicrobial and anti-carcinogenic properties [13]. As antioxidants, these compounds can protect cellular constituents against oxidative damage, thus limiting the risk of various degenerative diseases associated with oxidative stress [14]. They are able to neutralize free radicals by donating an electron or hydrogen atom [15]. In addition, phenolic compounds can act by increasing the activity of endogenous antioxidant enzymes such as GSH-PX, SOD or CAT and also by inhibiting the activity of enzymes such as xanthinase.

Structurally, phenolic compounds contain one or more aromatic rings with hydroxyl groups, encompassing a wide variety of chemical structures, from simple molecules to polymeric compounds [16].

I.4. General notions and methods of determination of chlorogenic acid, catechin, rosmarinic acid and verbascoside – the compounds of interest of this research paper

I.4.1. Chlorogenic acid

CGA has been extensively studied because it is one of the main phenolic compounds in the human diet, having many health benefits. The compound is found in foods and herbs such as apples [17], artichokes [18], carrots [19], coffee beans [20], potatoes [21], grapes [22], tobacco leaves [23], tea [22], wormwood [24] and honeysuckle [25].

Chemically, CGA is an organic phenolic acid compound, being an ester of caffeic acid and (L)-quinic acid.

In recent years a number of health benefits have been associated with the consumption of CGA. Among them, the following stand out:

- modulation of glucose metabolism in humans. Therefore, CGA has an antidiabetic effect [26] on type 2 diabetes by improving insulin action.
- prevents cataract development due to its anti-diabetic effect. This was indicated by the results of the studies carried out on laboratory animals.
- reduces the relative risk of cardiovascular disease, improving human vaso-reactivity. It also has antihypertensive properties [27].
- reduces the risk of gallstones.

I.4.2. Catechin

Catechins or flavonol-3-oles constitute a group of compounds that contain about 70% of the total polyphenols present in tea leaves. The catechins present in the green tea leaves are eight, as follows (-) epigallocatechin (EGC), (-) epigallocatechin gallate (EGCG), (-) epicatechin gallate (ECG), (-) epicatechin (EC), (-) catechin (C), (-) gallocatechin (GC), catechin gallate (GC) and gallocatechin gallate (GC) [28].

Catechins have many beneficial effects by capturing free radicals and delaying the degradation of the extracellular matrix induced by ultraviolet radiation and pollution [29]. Catechins have a direct protective action on the skin by activating collagen synthesis and inhibiting the production of metalloproteinases enzymes [30]. Due to the hydroxyl in the gallate group, EGCG and ECG are highly effective free radical traps compared to many other standard antioxidants such as ascorbic acid, tocopherol and trolox [31], [32], [33]. Thanks to these useful actions, catechins from tea are increasingly used in medical, pharmaceutical and cosmetic products and are actively studied in a wide variety of approaches.

I.4.3. Rosmarinic acid

Rosmarinic acid was first isolated by Scarpati and Oriente in 1958 [34] and is found in the composition of medicinal plants of the Laminaceae family, including rosemary (Rosmarinus officinalis), mint (Mentha spicata), sage (Salvia officinalis), rosemary (Melissa officinalis), chard (Origanum) and gimbrum (Origanum) [35], [36], [37].

Chemically it is an ester of caffeic acid and 3.4-dihydroxyphenyl lactic acid [38], containing in its structure two catecol fragments, therefore having two pairs of ortho-hydroxyl groups grafted on two aromatic cycles [39], easily oxidized, responsible for the electroactivity of this compound [40].

Rosmarinic acid has a remarkable medicinal value for the human body through its antioxidant [41], anti-inflammatory [42], anti-tumor [43], immunomodulatory [44], and

antimicrobial actions [45]. At the same time, the compound attenuates the T-lymphocyte receptors, with an effect on limiting allergic conditions such as conjuctivitis, rhinitis or allergic asthma, but it is also a protector against neurotoxicity, which can delay the development of Alzheimer's disease [46].

I.4.4. Verbascoside

Verbascoside, a derivative of hydroxyenemy acid, is found in abundance in olive oil, representing one of the most powerful antioxidants in this product, its unique antioxidant activity being due to the synergistic effect of the combination of the two diphenolic constituents, namely caffeic acid and hydroxytyrosol [47].

More concentrated in the olive fruit, but also present in the leaves, verbascoside has an important free radical-capturing activity and therefore has a direct impact on skin health, preventing oxidative damage associated with wrinkle formation, skin thinning or dehydration [48].

CHAPTER II. DETERMINATION OF ANTIOXIDANT ACTIVITY BY PHYSICAL AND CHEMICAL METHODS

The techniques and tools used to measure antioxidant activity have advanced remarkably in recent decades. The first methods performed measure the effectiveness of antioxidants against the formation of certain chemical species produced at oxidation and are therefore based on the measurement of lipid oxidation [49]. To date, various chemical tests coupled with highly sensitive and automated detection technologies are used to assess antioxidant activity through special mechanisms, e.g. neutralization of certain types of free radicals or ROS and metal chelating, among others [50]. Oxidation substrates or target samples have also been extended from food pattern systems to chemical compounds, biological materials, cell lines and even living tissues [51].

The methods for evaluating antioxidant activity are divided into three distinct categories, namely spectrophotometric methods, electrochemical and chromatographic tests. Depending on the chemical reactions involved, spectrometric methods fall into two categories: Hydrogen transfer reaction tests (HAT) and single electron transfer reaction tests (SET). Antioxidants can neutralize radicals or other ROS (e.g. hydrogen peroxide and lipid peroxide) through HAT and SET leading to the same end results, regardless of the mechanism involved, although kinetics and side reactions may vary [5]. Set and HAT reactions coupled with protons can also occur in parallel, and in this case the dominant mechanism in a given system depends on the antioxidant structure and properties, solubility and partition coefficient, as well as the solvent system [52].

II.1. HAT tests

The tests based on the transfer of the hydrogen atom measure the ability of an antioxidant to remove the free radicals by donating a hydrogen atom. The HAT mechanisms of antioxidant action are proven in the following reaction where the hydrogen atom (H) of a phenol (ArOH) is transferred to a peroxyl radical:

$$ROO^{\bullet} + AH/ArOH \rightleftharpoons ROOH + A^{\bullet}/ArO^{\bullet}$$

where: the aryloxyl radical (ArO•) formed from the reaction of the phenol (ArOH, an antioxidant) with a peroxyl radical (ROO•) is stabilised by resonance and AH are the protected biomolecules.

An efficient phenolic antioxidant should react faster than the biomolecules (the protected molecule) with the free radicals in order to give a protective effect against their oxidation [51].

Typical examples of HAT-based tests include ORAC, HORAC, TRAP and TOSC assays.

II.2. SET tests

The tests based on the transfer of a single electron, also called electron transfer (ET) tests, detect the ability of an antioxidant to transfer an electron in order to reduce metallic ions, carbonyl groups and free radicals [53]. The SET mechanisms of antioxidant action may be summarised by the following reactions:

$$ROO^{\bullet} + AH/ArOH \longrightarrow ROO^{-} + AH^{\bullet+}/ArOH^{\bullet+}$$

$$AH^{\bullet+}/ArOH^{\bullet+} + H_2O \longleftrightarrow A^{\bullet}/ArO^{\bullet} + H_3O^{+}$$

$$ROO^{-} + H_3O^{+} \longleftrightarrow ROOH + H_2O$$

The spectroscopic SET tests, including the Folin–Ciocalteu test, FRAP test and CUPRAC test, measure the capacity of an antioxidant to reduce an oxidant, which changes colour when reduced. The colour change degree is correlated with the concentration of the total antioxidant capacity. Moreover, electrochemical and nanotechnological methods also belong to the category of ST-based tests [54].

II.3. Mixed Mode Tests (HAT/SET)

These mixed mode tests are generally based on the elimination of a stable chromophore (like ABTS and DPPH), where HAT, ET, and PCET mechanisms may play different roles in varied proportions, depending on the corresponding reaction conditions (such as pH and solvent) [55]. Mixed mode tests (HAT/SET) mainly include the ABTS/Trolox equivalent antioxidant capacity (TEAC) test, the DPPH radical neutralisation test, and the DMPD radical neutralisation test.

CHAPTER III. DETERMINATION OF ANTIOXIDANT ACTIVITY USING ELECTROCHEMICAL SENSORS AND BIOSENSORS

In recent years, particular attention has been paid to the analysis of antioxidants using enzyme sensors and/or biosensors due to some advantages that these methods have, namely high sensitivity, simplicity of use, ease of storage, quick responses, easy automation, portability and ease of miniaturization, making them suitable for on-site diagnosis, reducing the risk of interference resulting from destabilization of compounds [56], [57].

III.1. Electrochemical Sensors for Determining Antioxidant Activity

Electrochemical sensors for the determination of antioxidants were developed using various types of electrodes, transducers, and receptors. In some cases, nanomaterials were integrated so as to obtain improved performances, thus increasing their sensitivity, stability, and selectivity. The emergence and application of nanomaterials as an integral part of sensors had a visible impact on research. Nanomaterials are characterized by certain special thermic, mechanic, optic, electric, and magnetic properties, which depend on size and can be calibrated by the simple adjustment of shape, size, and degree of agglomeration [58]. These properties

themselves and the effect of size are essential, which is manifested by the increase in electrochemical activity compared to that of the corresponding raw material [59].

Electrochemical methods were used, with consideration of their advantages in relation to the possibility of rapidly proving the antioxidant activity of numerous organic compounds. The oxidation potentials, measured through CV, were used to compare the antioxidant power of various compounds, such as phenols, flavonoids, cinnamic acids, and tannins, frequently using a GCE [60].

III.2. Electrochemical Biosensors for Determining Antioxidant Activity

Monitoring antioxidant activity through electrochemical biosensors, based on the redox principle, has many advantages compared to the conventional chemical methods and is commonly used for the initial screening of antioxidants. This technology does not require chemical reagents or sophisticated solvents, nor does it require special treatment of samples. It offers extended and reproducible information about electrodynamic processes and ensures a rapid achievement of determinations [61].

Enzyme-based biosensors have several advantages related to the nature of the enzyme. They are highly selective for a particular substrate and, for a large number of substrate molecules, reactions can be catalyzed by a single enzyme molecule, resulting in an amplification of the effect and an increase in sensitivity [62]. The enzymes commonly used in developing biosensors belong to the oxidoreductase, hydrolase, or lyase categories. At present, a variety of proteases are used to determine antioxidants and to evaluate their activity through biochemical oxidation, followed by electrochemical reduction [63]. Tyrosinase [64], laccase [65], peroxidase [66] and other proteases with simple or complex enzymatic bindings [67] are among them. The electric coupling of oxidoreductase and the electrochemical transducer have excellent characteristics, and monitoring is achieved through controlling the enzyme reaction in real time [62]. Specific enzymes can be used efficiently for the selective identification of important target compounds in food quality control. Laccase and tyrosinase are the two enzymes which are most frequently used to monitor antioxidants, especially phenolic compounds [68].

PART II. PERSONAL CONTRIBUTIONS
SENSORS AND BIOSENSORS DEVELOPED FOR ANALYSIS OF CHLOROGENIC ACID, CATECHINEI, ROSMARINIC ACID AND VERBASCOSIDE FROM NUTRACEUTICALS, COSMETICS OR FOOD PRODUCTS

CHAPTER IV. MOTIVATION, PURPOSE AND OBJECTIVES OF THE DOCTORAL THESIS

The occurrence of degenerative processes is correlated, in molecular biology, with the existence of a harmful surplus of free radicals, promoters of oxidative processes harmful to the body. The existence in plants of compounds with antioxidant properties and high content in compounds that can capture free radicals (carotenoids, polyphenols, flavonoids, anthocyanins, unsaturated fatty acids, vitamins, enzymes and co-factors) also stimulated the interest in their use in prophylactic and curative phytotherapy.

Therefore, the study of antioxidants and their implications in various fields, from the food field to medicine and pharmacy is of major interest to the scientific community.

A healthy diet, based on fruits and vegetables, is the most effective way to get the antioxidants the body needs. However, the situation is different when it comes to antioxidant supplements. In some circumstances, antioxidant-based pharmaceutical formulations can cause more harm than good. For example, at high concentrations these products can be harmful by:

- Action in the form of pro-oxidants, thus increasing oxidation;
- Protecting both healthy and harmful cells (such as cancer cells);
- Reducing the benefits of exercise;
- The occurrence of undesirable side effects, such as nausea, vomiting, dizziness and headaches, can even reach toxic levels in the body.

Therefore, one of the aims of this doctoral thesis was, on the one hand, in the development and electrochemical characterization of the various sensors and biosensors obtained by chemical modification, and on the other hand, in the development and characterization of the various sensors and biosensors obtained by chemical modification. in determining their ability to detect and quantify various bioactive compounds with antioxidant activity. These substances of interest in the research came from various nutraceutical formulations and cosmetic products purchased from community pharmacies and also from food samples represented by different extra virgin olive oils. Some of these products had declared the quantitative content of the active substances in the composition, but for some, the quantitative content or concentration were not specified on the label, but only the existence of those substances as the basic active principles of those nutraceutical formulations. The aim was also to make a comparison of the results obtained with newly developed devices in the laboratory with the existing information, provided by the manufacturer, on the antioxidant content in the products.

The **general objective** of this doctoral thesis was the manufacture and characterization of new chemically modified sensors based on carbon nanomaterials, mediators of the transfer of electrons or peptides and enzymatic biosensors aiming at the detection of bioactive compounds with antioxidant properties from various nutraceutical formulations, cosmetics or food samples, using electrochemical methods as detection techniques.

Starting from the general objectives, a number of specific objectives have been established that have been achieved, as evidenced by the articles published in specialized journals with an important impact factor, as follows:

- The development of a biosensor based on carbon screen-printed electrodes modified with graphene, manganese phthalocyanin and the enzyme tyrosinase for the detection of chlorogenic acid from various nutraceutical formulations.
- The development of new sensors based on the enzyme lacase, supported by three electrodes, as follows: CNT/SPCE, GNP/SPCE and Cnt-GNP/SPCE, applied for the detection of catechin in various phytotherapeutic products based on green tea extract.
- Quantification of rosmarinic acid in cosmetic products with a new sensor developed on the basis of an octapeptide, fixed by means of a cross-linking agent on the surface of the SPCE modified with a composite film of GO.
- Assessment and quantification of verbascoside content in different samples of extra virgin olive oils with minimal sample preparation using a SPCE modified with a composite GO film on the surface of which a pentapeptide has been immobilized.

CHAPTER V. MATERIALS AND METHODS USED TO DEVELOP NEW SENSORS AND BIOSENSORS FOR THE DETECTION OF PHENOLIC COMPOUNDS WITH ANTIOXIDANT PROPERTIES

V.1. Materials

V.1.1. Working electrodes

In all studies SPCEs purchased from Metrohm DropSens (www.dropsens.com) (Oviedo, Spain).

In the first study, namely the one that aimed to electrochemically determine chlorogenic acid in nutraceuticals using voltammetric sensors, SPCEs were used, modified by the manufacturer with GPH, respectively GPH and GNP.

In the second study on the detection of chlorogenic acid in nutraceuticals using a newly developed enzyme biosensor based on the enzyme tyrosinase, a manufacturer-modified SPCE with GPH was used. The electrode modification for the preparation of the biosensor was done, at first with a solution of MnPc 10^{-5} M (in chloroform), and at the next stage with a solution of Tyr enzyme of 3.50 mg/ml concentration in PBS 10^{-1} M (pH=7.0).

For the comparative study on the assessment of the antioxidant activity of catechin in nutraceutical products by electrochemical method and classical analytical methods, three biosensors obtained in the laboratory by modification of three SPCEs, namely CNT/SPCE, GNP/SPCE and Cnt-GNP/SPCE, were used. They were modified by immobilization of the Lac enzyme followed by reticulation with glutaraldehyde, thus achieving the three biosensors: CNT-Lac/SPE, GNP-Lac/SPE and CNT-GNP-Lac/SPE.

In the fourth study, namely the voltammetric detection of rosmarinic acid in cosmetics with a new electrochemical sensor based on C, go, modified with an octapeptide solution, to obtain the new sensor was used as a support for SPCE/GO. The subsequent modification in the laboratory involved adding octapeptide solution to the sensor surface, followed by reticulation, thus achieving the GO-peptide/SPCE sensor.

In the last study conducted to quantitatively determine and evaluate the antioxidant activity of verbascoside in olive oil by using a new electrochemical sensor based on C, GO, modified with a pentapeptide solution, SPCE was used with a GO film (SPCE/GO) to obtain the newly developed sensor (SPCE/GO). The 10 mg/ml pentapeptide solution has been added to its surface.

V.1.2. Electrochemical devices and cells

Two potentiostates were used to record, characterize and optimize electrode signals, namely potentiostat/galvanostat EG&G (Princeton applied Research, Oak Ridge, TN, USA), Model 263, Controlled by ECHEM software and biological potentiostat/galvanostat SP 150 (Bio-Logic Science instruments SAS, France) coupled with EC-Lab Express software operating in Windows. For both potentiostates, the electrochemical cell in which the electrodes were immersed had a capacity of 50 ml. The electrochemical measurements were performed using a conventional system containing three electrodes, namely an AG/AgCl/KCl_{3M} reference electrode (Princeton, applied Research), an auxiliary electrode consisting of a platinum wire and a working electrode, respectively SPCEs.

The FT-IR spectra were acquired with a Bruker ALPHA FT-IR spectrometer (BrukerOptik GmbH, Ettlingen, Germany) using THE OPUS software (BrukerOptik GmbH, Ettlingen, Germany) in the wave number range located between 4000 and 500 cm^{-1} (32 scans, 4 cm^{-1} resolution), Using the attenuated total reflectance method (ATR) as the sample exposure mode.

In the case of UV-vis spectrophotometric methods, the absorbances were measured using a Rayleigh UV2601 UV/vis double-beam spectrophotometer (Beijing Beifen-Ruili analytical Instrument, Beijing, China).

To analyze the surface morphology of enzyme biosensors, an electronic scanning microscope (FlexSEM 1000 II Hitachi, Japan) was used.

Fluorescence (emission) spectra were recorded using a FP-8350 Spectrofluorometer (JASCO, Tokyo, Japan).

For chromatographic analysis of phenolic compounds studied from various nutraceuticals, the U-HPLC-Q-Exactive Obitrap HRMS, controlled by Xcalibur software, version 4.1, was used.

The following were used to weigh the substances and prepare the solutions to be analyzed: Analytical balance Partner as 220/C/2 (S.C. Partner Corporation SRL, Bucharest, Romania) (Figure V.16), pipettes and micropipettes, quoted balloons and graduated cylinders.

V.1.3. Solutions and reagents

The reagents used in this study were purchased from Sigma-Aldrich (St. Louis, USA) and use without additional purification. For substances that required ultrapure water dissolution, it was obtained from the Mili-Q system (Millibore, Bedford, MA, USA).

In the first study to detect chlorogenic acid using electrochemical sensors, the following reagents were required: Potassium chloride, potassium ferrocyanide, sodium diphosphate and phosphoric acid. In preliminary experiments, the solutions of potassium ferrocyanide 10^{-3}M $K_4[Fe(CN)_6]$ - PBS 10^{-1}M were used. To obtain the 10^{-3} M chlorogenic acid stock solution used in electroanalytical studies, adequate amounts of chlorogenic acid were dissolved in PBS solution at pH = 7.0.

The following compounds were also used for interference studies, namely ferulic acid, vanilic acid and L-ascorbic acid (purchased from Riedel-de-Haën, Seelze, Germany).

At the same time, three food supplements were analyzed: Green Coffee Extract (Rotta Natura), Green Coffee (Pro Natura), Green Coffee Fit (Pro Natura).

For the second study of chlorogenic acid determination by biosensors, the following reagents were used: Sodium diphosphate and phosphoric acid for the preparation of PBS 10^{-1}M solution, chlorogenic acid, MnPc, mushroom tyrosinase (7164 U/mg).

The Folin–Ciocalteu reagent and a 15% sodium carbonate solution were used to validate the electroanalytical method using the Folin–Ciocalteu spectrophotometric method.

The DPPH 0.1 mm stock solution was prepared by weighing 0.0018g DPPH reagent and dissolving in 50 mL 96% ethanol.

The compounds used for interference studies were: ferulic acid, vanilic acid, p-coumaric acid and L-ascorbic acid.

The nutraceutical products used in the analysis were purchased from natural stores, the pharmaceutical form of presentation for all three products being the capsule.

In the comparative study on the evaluation of the antioxidant activity of catechin in nutraceutical products using a newly developed electrochemical method and spectrophotometric methods of analysis, reagents were used: Sodium acetate, glacial acetic acid, hydrochloric acid and catechin. Mushroom lacquase (Trametes versicolor) had a concentration of 0.78 U/mg, with which a solution of 2 mg/mL was obtained in ABS 10^{-1} M (pH=5.2).

For the determination of antioxidant activity by spectrophotometric methods, the following stock solutions were used: DPPH 0.1 mm solution obtained by weighing 0.0036g DPPH reagent, followed by dissolution in 100 mL 96% ethanol, Galvinoxil 0.1 mm solution prepared by weighing 0,0042g galvinoxyl reagent followed by dissolution in 100 mL 96% ethanol and concentrated solution of radical cation $ABTS^{\cdot+}$, obtained by weighing 0,0128g ABTS reagent and 0,0033g $K_2S_2O_8$, both subsequently dissolved in 50 mL ultrapure water.

To validate the results obtained by the modified electrodes, three products containing the substance of interest, namely catechin, were selected.

For the fourth study, the following reagents were needed: Potassium chloride, potassium ferrocyanide, potassium ferricyanide, sodium diphosphate, phosphoric acid and rosmarinic acid. Octapeptide of purity >95% was purchased from ProteoGenix, Schiltigheim, France. The purity of the peptide and its retention time have been confirmed by RP-HPLC chromatography on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, USA). To immobilize the peptide on the sensor surface, a solution obtained from 10 mg/mL peptide in PBS 10^{-1} M (pH=6.5) was used.

The cosmetic products used in the analysis to validate the results obtained by the proposed method were purchased from natural stores, the pharmaceutical form of presentation for all three products being cream. The products have a diverse composition of active principles and excipients, and on the label of each is indicated the presence of extract from rosemary leaves (Rosmarinus officinalis).

In the last study conducted to quantitatively determine verbascoside in olive oil, the following reagents were required: monosodium phosphate, disodium phosphate, verbascoside powder of analytical purity, pentapeptide, purity >95% and compounds structurally similar to verbascoside (oleuropein, thyroid and hydroxytyrosol) used for interference studies.

For the study of the sensitive properties of the sensor, 10 extracts from different EVOO obtained by liquid-liquid extraction were analyzed [69].

V.2. Methods of characterization of electrodes, electroactive mediators and biologically active compounds with antioxidant properties

V.2.1. Electrochemical methods

Electrochemical techniques with controlled potential (potentiostatic methods) have been applied in qualitative and quantitative analysis of polyphenolic compounds. Basically, the electrode potential causes redox reactions on the species of interest and the resulting current is subsequently measured [70]. This generated current is proportional to the concentration of an electroactive species. Potential hostages include CV, SWV and DPV, electrochemical methods experienced in this paper.

V.2.1.1. Analysis by CV

CV is an efficient and versatile electroanalytical technique that allows to investigate the mechanism of the redox and transport properties of a system in solution. In the CV, the potential of an electrochemical system is changed back and forth between two potential limit values, and the current response is measured relative to the potential. Voltammograms are graphical representations of the working electrode current according to the counter-electrode current [71].

V.2.1.2. Analysis by SWV

SWV is one of the fastest and most sensitive voltametric techniques for determining electroactive organic compounds due to very low non-pharadaic current [72]. The limits of detection can be compared to those of chromatographic and spectroscopic techniques. In addition, the analysis of the characteristic parameters of this technique also allows the assessment of the kinetics and process mechanism of the studied electrodes [73]. At the same time, the peaks corresponding to oxidation or reduction of the electroactive species at the electrode surface can be obtained in the same experiment, and the reversibility of the electron transfer can be examined in a single scan, the current being measured both during negative and positive impulses [74].

V.2.1.3. Analysis by DPV

DPV is another widely used electrochemical technique, suitable for characterizing the redox behavior of antioxidants [75]. In DPV, the potential is scanned in a step form, with the height and width of the fixed step, but with an additional pulse applied. In general, the current is measured before and after the application of a pulse [76]. The difference between these two currents minimizes capacitive current, leading to voltamograms with higher signal/noise ratios [77]. Another feature of the DPV is its higher resolution than that achieved by the CV. For this reason, DPV is appropriate when greater selectivity is required [78].

V.2.2. EIS analysis

EIS is a technique used in the analysis of electrochemical processes that take place at the electrode/electrolyte solution interface [79]. This is a method of identifying and determining parameters in a model developed based on the frequency of the response of the electrochemical system studied. In such experiments, a response frequency analyzer coupled

with an electrochemical interface is used, which measures the current response of the system as it changes the frequency of an input sine signal that is applied to an unknown sample [80].

The results of this analysis are expressed through a Bode graph or a Nyquist graph [81].

V.2.3. HPLC analysis

Liquid chromatography is an important technique for separating the chemicals in the mixture, resulting in the qualitative and quantitative determination of the components in that mixture. This method of separation has as principle the varied distribution between two phases, namely a stationary phase and a moving phase of the components of the mixture [82].

V.2.4. FT-IR analysis

The FT-IR method is a non-invasive analytical technique often used in biology and medicine, as it allows the rapid obtaining of a biochemical fingerprint of the investigated sample, providing information about the content of biomolecules [83]. This spectroscopic tool is successfully applied not only for the study of the structural properties of isolated biomolecules such as proteins, nucleic acids, lipids and carbohydrates, but also for the characterization of complex biological systems such as intact cells, tissues and whole organisms [84].

V.2.5. SEM analysis

SEM is a non-destructive analysis technique that uses an electron beam probe to analyze surface details up to the nano scale [85]. The scanning electron microscope produces high-resolution images, making them suitable for a wide range of applications in many fields of science, industry and medicine [86]. Thus, recent research into cellular morphology, biocompatible material development, tissue engineering research and microbiology is based on advanced SEM imaging techniques [87].

CHAPTER VI. ELECTROCHEMICAL DETERMINATION OF CHLOROGENIC ACID IN NUTRACEUTICALS USING VOLTAMMETRIC SENSORS BASED ON CARBON NANOMATERIALS

The purpose of this study was to develop and characterize new SPCEs modified with GPH and GNP and to establish their ability to determine the qualitative and quantitative determination of CGA. The electroanalytical method will also be validated for quantifying this bioactive compound of interest in various nutraceutical products using a classical method, namely FT-IR.

VI.1. Preliminary Studies

The behavior of the three electrodes - C-SPE, GPH-SPE, and GPH-GNP-SPE - in a 10^{-1} M PBS solution, at pH = 7, optimum potential range between −0.4 V and +1.3 V, was studied during the first stage. Following the measurements, no peaks were registered in the potential field analyzed, which demonstrates the high purity of the materials used for the electrodes and the lack of active surface contamination. It was noticed that, in the case of the GPH-GNP-SPE electrode, the base currents were reduced, which represents an advantage of modifying the sensor surface with GPH and GNPs.

VI.2. Electrochemical Properties of the Electrodes in Electroactive Solution

During the next stage, the electrochemical behavior of SPEs in a solution containing 10^{-3} M $K_4[Fe(CN)_6]$ dissolved in 10^{-1} M PBS, pH = 7, was analyzed using CV as the detection method [42]. The potential field in which the signal proved to be stable was between –0.4 and +0.7 V. In view of stabilizing the sensors, 5 cycles were registered in the solution to be analyzed.

The main parameters obtained from CVs are shown in Table VI.1.

Table VI.1. *The main features of electrodes obtained by cyclic voltammetry*

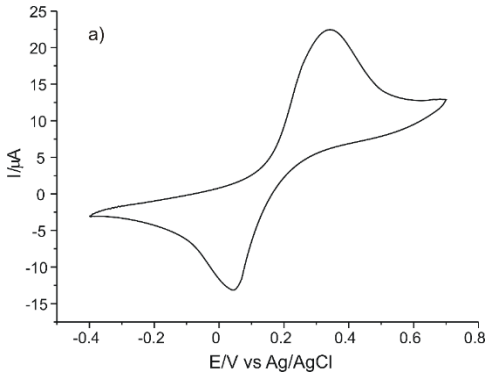
Sensor	Ipa (μA)	Ipc (μA)	Ipc/Ipa	Epa (V)	Epc(V)	$E_{1/2}$ (V) (Epa-Epc)	ΔE_p (V) (Epa-Epc)
C-SPCE	23,88	-26,04	1,09	0,447	0,001	0,224	0,446
GPH-SPCE	36,59	-40,51	1,10	0,286	0,159	0,222	0,127
GPH-GNP-SPCE	81,42	-87,27	1,07	0,298	0,109	0,203	0,189

The highest current values were obtained for GPH-GNP-SPE, with values close to those obtained for GPH-SPE and somehow different from those obtained for C-SPE. Taking these results into account, the greatest sensitivity in detecting the ferrocyanide ion was obtained in the case of GPH-GNP-SPE (where the values for Ipa and Ipc are the highest).

VI.3. Electrochemical Responses of Sensors in CGA Solution

The electrochemical behavior of sensors in CGA solution was studied by means of CV.

To obtain a stable answer from the sensor, five cycles were necessary in the optimized potential field (from −0.4 V to +0.7 V). The CVs presented in figure VI.1 were obtained after signal stabilization.



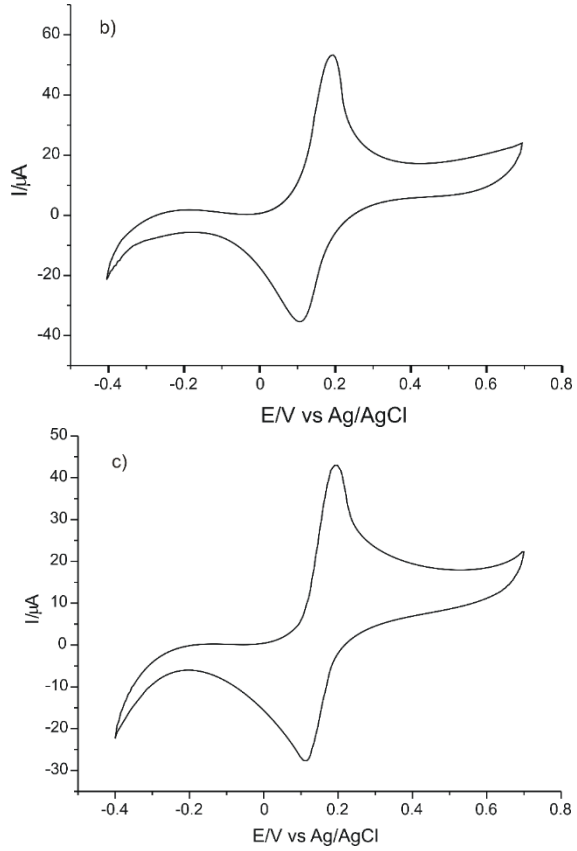


Figure VI.1. *CVs of of (a) C-SPEs, (b) GPH-SPE, and (c) GPH-GNP-SPE immersed in 10^{-3} M CGA solution (support electrolyte 10^{-1} M PBS solution). Scan rate 0.1 V·s^{-1}.*

What may be noticed in all three CVs is the occurrence of an anodic peak corresponding to the oxidation process and of a cathodic peak corresponding to the reduction process. Since the CGA contains two OH groups in orto position, by oxidating this compound, the respective quinone is formed, releasing two electrons and two protons [88].

Table VI.2 illustrates the most important parameters obtained from the CVs or calculated depending on the experimental parameters.

Table VI.2. *The values of the parameters obtained from the CVs of all the electrodes immersed in 10^{-3} M CGA solution (the electrolyte support was 10^{-1} M PBS of pH = 7).*

Sensor	Epa(V)	Ipa(µA)	Epc(V)	Ipc(µA)	Ipc/Ipa	ΔE_p (V)
C-SPCE	0,279	21,08	0,042	-13,17	0,62	0,237
GPH-SPCE	0,197	53,50	0,114	-36,69	0,68	0,083
GPH-GNP-SPCE	0,189	43,23	0,113	-28,01	0,65	0,076

All three electrodes studied showed two clear, well-defined peaks and a quasi-reversible behavior, as indicated by the ΔE (V) value and the Ipc/Ipa ratio.

VI.4. Calibration Curve and Determination of Detection Limit

The CVs in CGA solutions of various concentrations were recorded using C-SPE, GPH-SPE, and GPH-GNP-SPE. The calibration curves were achieved in the concentration field of 0.1–1.20 µM. The 10^{-1} M PBS (pH = 7) solution was used as supporting electrolyte, and the CGA stock solution had a concentration of 10^{-3} M. The scanning rate was 0.1 $V \cdot s^{-1}$ in each case, and the potential field was situated between −0.4 V and +0.7 V.

The variation of the sensor response depending on the concentration of the CGA solution is presented in Figure VI.2a. As may be seen, the intensity of the anodic and cathodic peaks increases with the increasing concentration.

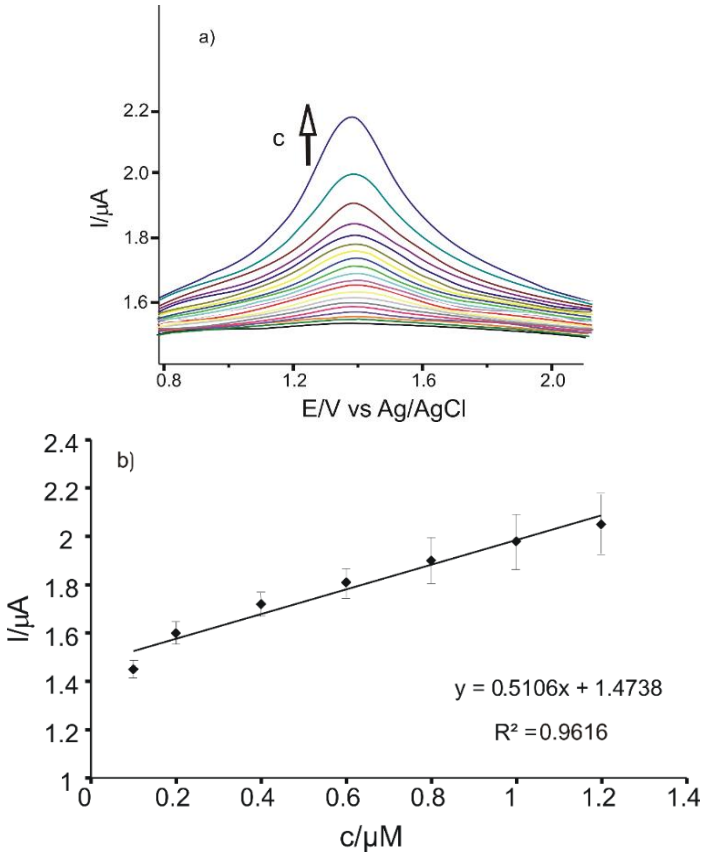


Figure VI.2. a) *Zoomed-in view of the anodic peak zone of the CVs registered with GPH-GNP-SPE immersed in CGA solutions with the concentrations in the range 0.1–1.20 µM; b) linear dependence between the anodic peak current and the concentration of the CGA solution.*

Table VI.3 shows the results obtained for LOD and LOQ, calculated for the three sensors used in this study.

Table VI.3. *LOD and LOQ values for CGA detection for each of the three sensors*

Sensor	LOD (M)	LOQ (M)
C-SPCE	$6{,}50 \times 10^{-7}$	$2{,}16 \times 10^{-6}$
GPH-SPCE	$0{,}73 \times 10^{-7}$	$2{,}45 \times 10^{-6}$
GPH-GNP-SPCE	$0{,}62 \times 10^{-7}$	$1{,}94 \times 10^{-7}$

The lowest detection limit was obtained for GPH-GNP-SPE. Consequently, the sensitivity of the sensors decreased in the following order: GPH-GNP-SPE > GPH-SPE > C-SPE.

VI.5. Quantitative Determination of CGA in Nutraceutical Products

The following analysis consisted in determining CGA from real samples, with three commercial products being selected—namely, Green Coffee Extract (Rotta Natura), Green Coffee (Pro Natura), and Green Coffee Fit (Pro Natura). These products were analyzed using two methods: CV (the method described in this study) and FTIR analysis (standard method) [89]. Therefore, the goal of this analysis was to compare the results obtained through the methods mentioned and to validate the electroanalytical method.

Table VI.4 shows the results expressed as a percentage.

Table VI.4. *CGA content determined in three food supplements by using voltammetric and spectrophotometric method.*

Nutraceutical product	Voltammetric method c% CGA	Spectrophotometric method c% CGA
Green Coffee Extract (Rotta Natura)	4,43	4,83
Green Coffee (Pro Natura)	4,38	4,62
Green Coffee Fit (Pro Natura)	5,86	6,43

The next step involved the application of another method for determining the polyphenolic content, namely UHPLC-MS/MS with ESI ionization, a high-resolution Q Exactive™ focus Hybrid Quadrupole-Orbitrap (ThermoFisher Scientific) mass spectrometer equipped with HESI coupled to a high-performance liquid chromatograph Ultimate 3000 UHPLC (ThermoScientific). Chromatographic separation was performed on a Kinetex® C18 column (100 × 2.1 mm, particle diameter 1.7 µm) at a temperature of 30 °C. An example of a chromatogram obtained with the chromatograph system in a real sample is shown in Figure VI. 3.

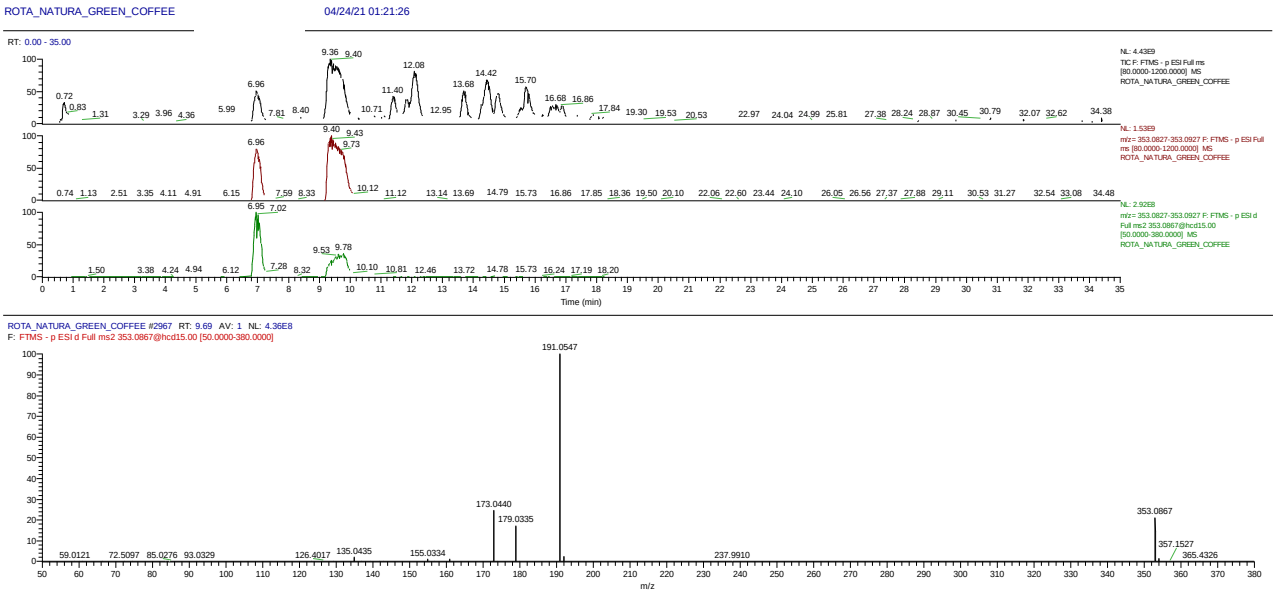


Figure VI.3. *Chromatographic analysis of CGA in Green Coffee Extract (Rotta Natura)*

From the data obtained, the percentage concentration of chlorogenic acid per capsule for each pharmaceutical product studied was calculated, the results given in Table VI.5.

Table VI.5. *The chlorogenic acid content determined in the samples analyzed using the HPLC method*

Nutraceuticals	HPLC method c% CGA
Green Coffee Extract (Rotta Natura)	4,18
Green Coffee (Pro Natura)	3,96
Green Coffee Fit (Pro Natura)	5,96

From Table VI.5 it can be seen that the values obtained by the chromatographic method are close to those obtained by the CV and FT-IR methods (according to Table VI.4), which proves once again the accuracy and sensitivity of the GPH-GNP-SPCE sensor, but also the feasibility of the voltammetric method.

VI.7. Conclusions

Of the three electrodes, GPH-GNP-SPCE proved to have a very good sensitivity and a very low limit of detection for CGA. The analytical method may be successfully applied in determining CGA from real samples. The results obtained with GPH-GNP-SPE are very close to those obtained through standard methods of analysis, indicating a trust level of 99%.

CHAPTER VII. DEVELOPMENT OF A NEW ENZYME BIOSENSOR BASED ON GRAPHENE, MANGANESE PHTHALOCYANIN AND TYROSINASE, WITH APPLICABILITY IN THE ASSESSMENT OF ANTIOXIDANT ACTIVITY OF CHLOROGENIC ACID IN NUTRACEUTICALS

The objective of this paper is to assess the electrochemical behavior of a new biosensor based on graphene, manganese phthalocyanin and tyrosinase (GPH-MnPc-Tyr/SPCE) when detecting CGA through various voltammetric techniques. At the same time, the electroanalytical method used to quantify CGA in three different nutraceutical products will be validated using the Folin-Ciocalteu method. This study also aimed to determine the DPPH radical reduction capacity of the three nutraceuticals analyzed.

VII.1. Preparation of the biosensor

VII.1.1. Tyrosinase – general notions

The use of the enzyme tyrosinase for the development of enzyme biosensors is of great interest in medical research, but also in industrial biotechnology, given the possibility of combining micrometric or nanoscale-sized materials with certain biomolecules.

VII.1.2. Preparation of enzyme biosensor by immobilizing the enzyme tyrosinase

VII.1.2.1. Preparation of the GPH-MnPc/SPCE Biosensor

To prepare the modified GPH-MnPc/SPCE biosensor, several stages were covered. Initially, 10 µL of 10^{-5} M manganese phthalocyanine solution in chloroform was added - sequentially, with pauses for drying, through the drop-and-dry technique - to the surface of the GPH/SCPE. Drying was performed at room temperature for 30 min. An Eppendorf micropipette was used to add the manganese phthalocyanine solution.

VII.1.2.2. Preparation of the GPH-MnPc-Tyr/SPCE Biosensor

To prepare the biosensor, GPH-MnPc/SPCE was used as the support. Using the drop-and-dry technique, 10 µL were added sequentially, in two stages (5 µL in each), with a 3 h pause for drying in between. The enzyme reticulation was achieved by maintaining the sensor above a container with 5 mL of 2% glutaraldehyde, for 1 min.

VII.2. Preliminary Studies to Characterise the Electrode

The surface morphology of the biosensor GPH-MnPc-Tyr/SPCE has been characterized by SEM. Thus, Figure VII.1 shows an image obtained through SEM of the morphology of the surface of the composite nanofilum containing graphene, manganese phthalocyanin and tyrosinase, where graphene nanosheets and other components are well highlighted.

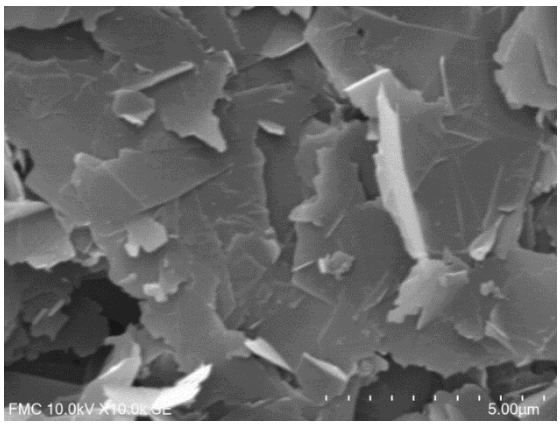


Figure VII.1. *SEM picture of the GPH-MnPc-Tyr/SPCE surface*

VII.3. Electrochemical Properties of Electrodes in CGA Solution

The qualitative and the quantitative determination of CGA was carried out through CV and SWV. These methods are useful in understanding the electrochemical processes which take place on the surface of the electrodes. GPH-MnPc/SPCE and GPH-MnPc-Tyr/SPCE electrodes were used at this stage of the study and the scan rate was 0.1 V × s^{-1}. To obtain a stable response of the electrodes, three cycles were necessary for the optimised potential range (from −0.4 V to 0.7 V).

Figure VII.2 shows the stable responses of the modified working electrodes at each stage of modification, immersed in CGA 10−3 M PBS 10^{-1} M solution (pH = 7.0)

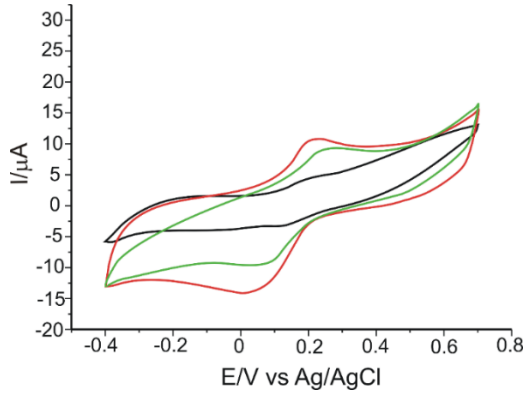


Figure VII.2. *CVs of GPH-MnPc/SPCE (black line), GPH-Tyr/SPE (green line), and GPH-MnPc-Tyr/SPCE (red line) immersed in 10^{-3} M CGA solution (support electrolyte 10^{-1} M PBS solution). Scan rate 0.1 V × s^{-1}.*

All three voltammograms highlighted an anodic and a cathodic peak of different intensities and potentials, corresponding to the redox processes of CGA at the electrode surface. In the case of the GPH-MnPc-Tyr/SPE biosensor, peaks are the most intense, and the cathodic peak appears at lower potential values as compared to the GPH-Tyr/SPE biosensor, which does not have a mediator in the sensing layer. Therefore, the mediator used, MnPc, facilitates the electron exchange and has a positive electrocatalytic effect, lowering the potential necessary for the reduction of CGA quinonic derivative, enzymatically and/or electrochemically formed

The electrochemical parameters obtained for all the (bio)sensors developed in this study are presented in Table VII.1.

Table VII.1. *The values of the parameters were obtained from the cyclic voltammograms of all the electrodes immersed in 10^{-3} M CGA solution (the electrolyte support was 10^{-1} M PBS of pH = 7.0).*

Sensor	Epa (V)	Ipa (μA)	Epc (V)	Ipc (μA)	Ipc/Ipa	ΔE (V)
GPH-MnPc/SPCE	0,190	4,30	0,117	-3,22	0,74	0,073
GPH-Tyr/SPCE	0,283	7,65	0,095	-10,20	1,33	0,188
GPH-MnPc-Tyr/SPCE	0,214	10,81	0,017	-14,14	1,30	0,197

The two electrodes, GPH-MnPc/SPCE and GPH-MnPc-Tyr/SPCE were used to record the square-wave voltammograms in 10^{-3} M CGA solution (support electrolyte 10^{-1} M PBS of pH = 7.0). The potential range studied was between −0.4 V and +0.7 V, with a pulse height of 0.10 V, an increment of the potential of 7 mV, and a frequency of 15 Hz. Through this method, similar results to those of the cyclic voltammetry were obtained. With both of the electrodes, there was a highlighted intense and better-defined reduction peak and a reduced background current. The square-wave voltammograms are presented in Figure VII.3.

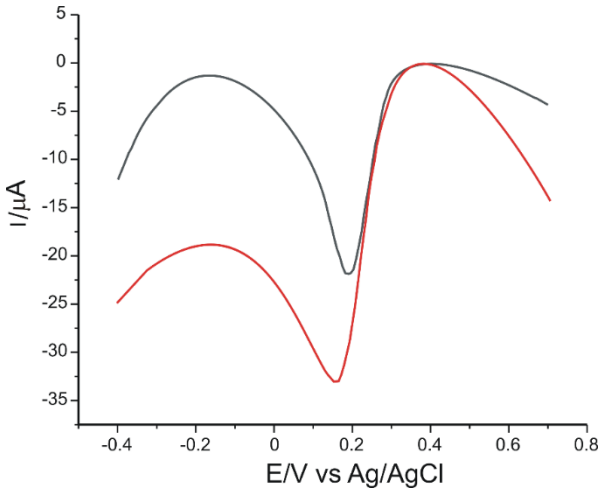


Figure VII.3. *Square-wave voltammograms obtained for GPH-MnPc/SPCE (black line) and GPH-MnPc-Tyr/SPCE (red line) by immersion in 10^{-3} M CGA solution (10^{-1} M PBS electrolyte pH = 7.0). The potential range was between −0.4 and +0.7 V, pulse height 0.10 V, and a potential increment of 7 mV at a frequency of 15 Hz.*

VII.4. Influence of Scan Rate on the Voltammetric Response

The next stage consisted of the study of the electrochemical behaviour of the two electrodes in 10^{-3} M CGA solution (the supporting electrolyte was 10^{-1} M PBS of pH 7.0), applying scan rates between 0.1–1.0 V × s^{-1}.

Figure VII.4 presents the CVs of the two modified electrodes recorded for different scan rates.

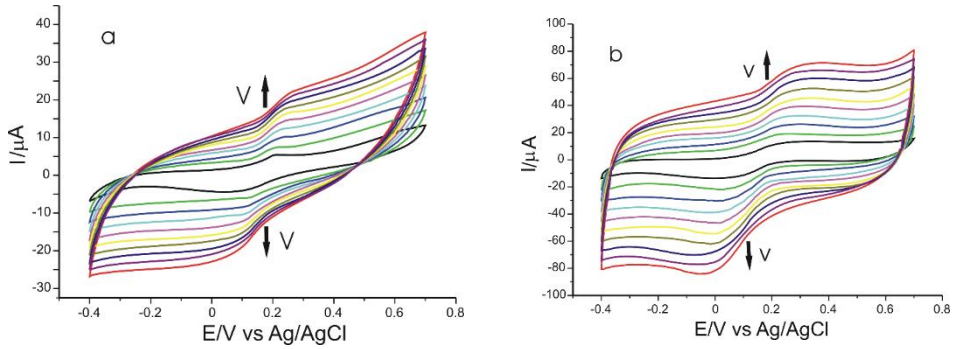


Figure VII.4. *CVs of GPH-MnPc/SPCE (a) and GPH-MnPc-Tyr/SPCE (b) recorded at various scan rates within the range 0.1–1.0 V × s^{-1}*

Figure VII.5. presents the linear dependence between the cathodic peak currents and the scan rates in the case of the two modified electrodes.

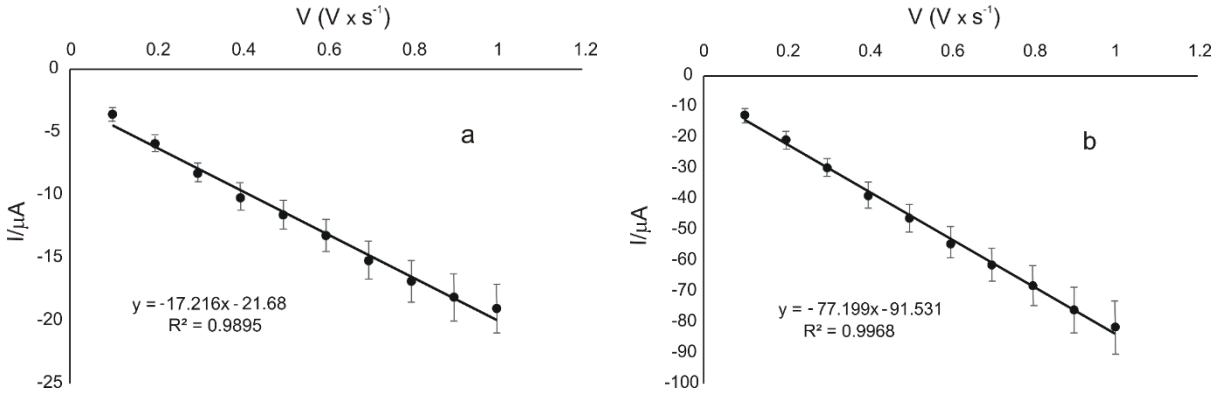


Figure VII.5*. Linear dependence between the cathodic peak currents (Ipc) and the scan rates for GPH-MnPc/SPCE (a) and GPH-MnPc-Tyr/SPCE (b)*

The coverage degree of the electrode surface with the electroactive species (Γ) was calculated using the equation of the dependence between the current of the cathodic peak and the scan rate, according to the Laviron equation [90]. The results obtained are presented in Table VII.2.

Table VII.2. *The linear fitting equations (Ipc vs. v), R2, and Γ for both electrodes used in the electroanalysis*

Electrode	Equation	R^2	Γ (mol·cm^{-2})
GPH-MnPc/SPCE	Ipc = 17,216 × 10^{-5} v (V × s^{-1}) – 21,68 × 10^{-5}	0,9895	3,34 x 10^{-11}
GPH-MnPc-Tyr/SPCE	Ipc = 77,199 × 10^{-5} v (V × s^{-1}) – 91,53 × 10^{-5}	0,9968	1,50 x 10^{-10}

The Γ values obtained for CGA with the two modified electrodes are comparable with those obtained for the other tyrosinase-based biosensors used to detect phenolic compounds and are reported in the literature [64], [91], [92], [93].

VII.5. Calibration Curve and Limit of Detection

To obtain the calibration curve, cyclic voltamograms of CGA have been recorded when varying quantities of CGA 10^{-3} M stock solution in 50 ml PBS 10^{-1} at pH 7.0 have been added successively, between 5 µl and 1000 µl. Each step of adding volumes was followed by agitation. After homogenization of the solution to be analyzed, cyclic voltamograms were recorded. The concentration range studied was 0.1 µm – 10.48 µm.

Table VII.3 shows the results obtained for the LOD and LOQ calculated for the biosensor GPH-MnPc-Tyr/SPCE used in this study.

Table VII.3. *Equation of linear dependence between Ipc and c, R2, LOD, and LOQ for GPH-MnPc-Tyr/SPCE*

Electrode	Equation	R^2	LOD (M)	LOQ (M)
GPH-MnPc-Tyr/SPCE	Ipc= -0,0692c – 9,3141	0,9688	1,40 x 10^{-6}	4,69 x 10^{-6}

Due to the presence of tyrosinase, which facilitates the interaction with CGA and confers the biosensor selectivity and sensitivity, the latter has superior performance as compared to the sensor.

VII.6. Quantitative Determination of CGA in Nutraceutical Formulations

Three pharmaceutical products from the food supplements category were selected, namely Green Coffee Bean 400mg Jarrow formulas, Green Coffee Bean complex (Adams Vision) and Green Coffee (Biotech USA) and qualitatively analyzed using the FT-IR spectrometric method [89]. The FT-IR spectra for commercial products are shown in Figure VII.6.

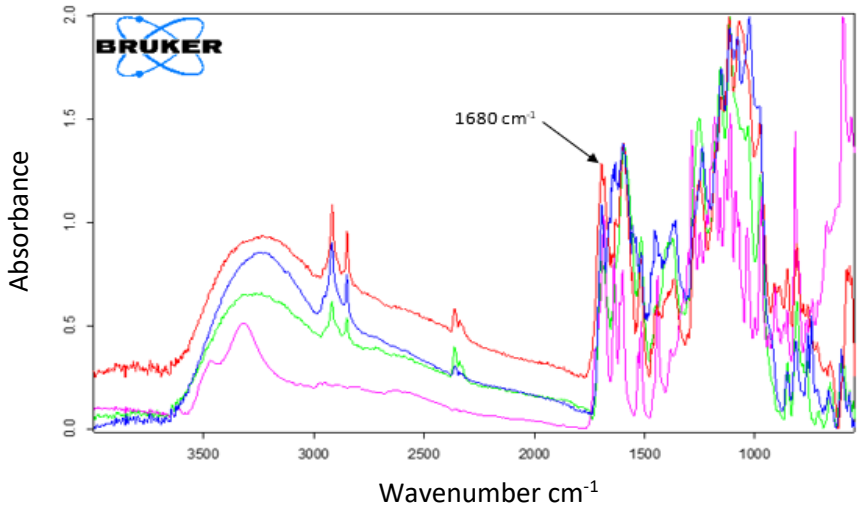


Figure VII.6. *FT-IR spectra of: Green Coffee Biotech USA (red line), Green Coffee Bean Complex Adams Vision (blue line), Green Coffee Bean 400mg Jarrow Formulas (green line), pure CGA (pink line)*

For the quantitative analysis of the three pharmaceutical formulations, two methods were employed: CV (the method developed in this study) and the Folin–Ciocalteu assay.

With regards to cyclic voltammetry, the measurements were recorded in the potential range situated between −0.4 V and 0.7 V. The kinetics of the redox processes was achieved by recording the cyclic voltammograms with a scan rate of 0.1 V × s^{-1}, using three different quantities from each product: 0.0050 g, 0.0070 g and 0.0090 g.

The three voltammograms indicate the appearance of an anodic peak and a cathodic peak at approximately the same potentials, as in the case of the standard CGA solution. Therefore, GPH-MnPc-Tyr/SPE has better selectivity and sensitivity to detect CGA in pharmaceutical products and nutraceutical products, respectively.

The results, expressed as percentage concentrations, are presented in Table VII.4.

Table VII.4. *CGA concentrations in nutraceuticals obtained by the voltammetric method, and by Folin–Ciocalteu spectrophotometric method, respectively.*

Nutraceutical	Voltammetric method c% CGA	Spectrophotometric method c% CGA
Green Coffee Bean Complex (Adams Vision)	83,72	87,39
Green Coffee (Biotech USA)	79,77	80,21
Green Coffee Bean 400mg Jarrow Formulas	79,72	80,11

VII.7. Stabilitatea, reproductibilitatea și repetabilitatea biosenzorului

The stability of the GPH-MnPc-Tyr/SPCE biosensor was evaluated through 30 measurements, carried out at regular intervals (1 day) for 1 month, using a CGA 10^{-3} M solution, through CV. During this period, the sensor was stored in a refrigerator at 4 °C. The results obtained did not highlight important differences between the cathodic currents recorded on different days; the variation coefficients being smaller than 5%, thus confirming that the biosensor is stable and can be used in the electroanalysis.

To verify the reproducibility of the proposed method, we have investigated the response of three biosensors, identically prepared in CGA 10^{-3} M solutions. No differences higher than 2% were noticed between the values of the cathodic current measured for the three biosensors.

The tests for the study of repeatability were carried out in a 50 µM CGA-10^{-1} M PBS solution. The value of the variation coefficient for the cathodic peak, determined in the seven consecutive determinations in the same solution, did not surpass 3%. Between measurements, GPH-MnPc-Tyr/SPCE was rinsed with a PBS solution of 10^{-1} M of pH = 7.0. Therefore, the biosensor can be used repeatedly to determine CGA.

VII.8. Interference Studies

In optimum experimental conditions, the influence of several related compounds, such as L-ascorbic acid, vanillic acid, ferulic acid, and p-coumaric acid, was studied, as these substances could interfere in electroanalysis. The detection technique used was CV. The biosensor signal was determined in the absence and in the presence of the interferent, and the relative response was calculated in percentages. The results obtained show that the

cathodic peak corresponding to CGA does not change significantly in the presence of interferents. These are presented in Table VII.5.

Table VII.5. *Interference of chemically related compounds with the quantitative determination of CGA 10^{-5} M*

Interfering Compound	Concentration of the Interferents	Ratio	Recovery %	RSD %
-	-	-	100	0,50
L-ascorbic acid	10^{-5} M	1:1	97,45	1,82
Vanillic acid	10^{-5} M	1:1	99,5	0,60
Ferulic acid	10^{-5} M	1:1	97,24	1,97
p-Coumaric acid	10^{-5} M	1:1	96,81	2,28

Taking these results into account, it can be affirmed that the GPH-MnPc-Tyr/SPCE biosensor has adequate selectivity and precision for determining CGA in real samples..

VII.9. Determination of CGA Antioxidant Activity through DPPH Method

DPPH radical reduction capacity expressed as percentage was calculated according to the following equation [94]:

$$\% \text{ DPPH Reduction} = 100 \times (AD - AE/AD)$$

where AE is the absorbance of the solution when the pharmaceutical product has been added and AD is the absorbance of the blank DPPH solution.

The values (expressed in percentages) of DPPH reduction were normalised per tablet for all three pharmaceutical samples. All the tests were carried out in triplicate, and the results were reported as averages. It was noticed that DPPH scavenge depended on the CGA concentration in the samples. The results obtained for the capacity to scavenge the DPPH radical, expressed in percentages, are given in Table VII.6.

Table VII.6. *DPPH reduction capacity in the case of the three nutraceuticals*

Product	**Green Coffee Bean Complex Adams Vision**	**Green Coffee Biotech USA**	**Green Coffee Bean 400mg Jarrow Formulas**
% DPPH Reduction	91.66	91.05	93.07

It can be affirmed that CGA is active in all the three products, and is present in high concentrations, as mentioned by the producer on the label, and as such, has been determined through the analytical method employed in this study.

VII.10. Conclusions

The GPH-based screen-printed electrode, modified with MnPc and Tyr, was developed and used to study and determine the electrochemical behavior of CGA.

By using cyclic voltammetry and square-wave voltammetry as detection methods, remarkable results were achieved - with applicability in laboratory practice. The calibration curve of GPH-MnPc-Tyr/SPCE towards CGA showed linearity in the 0.1–10.48 µM concentration range and the values of LOD and LOQ were low, close to those obtained by other biosensors based on tyrosinase in the detection of phenolic compounds.

Moreover, the biosensor was applied for the quantitative detection of CGA in three nutraceutical formulations, through CV, employing the Folin – Ciocalteu spectrophotometric method for its validation, with similar results in both cases. Through the DPPH method, the antioxidant activity of the compound was also determined, thus demonstrating the antioxidant effect of CGA in the three nutraceutical products studied.

CHAPTER VIII. COMPARATIVE STUDY ON THE EVALUATION OF THE ANTIOXIDANT ACTIVITY OF CATECHINEI IN NUTRACEUTICAL PRODUCTS USING A NEWLY DEVELOPED ELECTROCHEMICAL METHOD AND SPECTROPHOTOMETRIC METHODS OF ANALYSIS

The purpose of this study is to develop easy-to-use enzyme sensors with fast response and high precision, applied for the detection of catechin in in various nutraceutical formulations containing green tea extract. These new devices were obtained by the enzyme varnish modification of three electrodes as follows: Carbon nanotubes based screen-printed electrode (CNT/SPCE), gold nanoparticle based screen-printed electrode (GNP/SPCE) and carbon nanotubes based screen-printed electrode and gold nanoparticles (Cnt-GNP/SPCE). The newly developed devices have been used to study the catechin content of nutraceutical formulations containing green tea extract using as a DPV detection method. In addition, the proposed electrochemical methods have determined the antioxidant activity of these products, making a correlation between the data obtained by electrochemical methods (CV and DPV) and those resulting from the application of spectrophotometric methods for determining antioxidant activity, such as DPPH, ABTS and galvinoxyl.

VIII.1. Preparation of enzyme biosensors by immobilizing the enzyme laccase

Three sensors, namely CNT/SPCE, GNP/SPCE and CNT-GNP/SPCE, were used as the substrate for biosensor preparation. Using a drop-and-dry technique, 10 µL Lac enzyme solution was added to each sensor, sequentially in two steps (5 µL in each step) with a 3 h drying break between the two steps. Enzyme cross-linking was performed by suspending each sensor over 5 mL 2% (v/v) glutaraldehyde for 1 min.

VIII.2. Characterization of the Biosensors

Two methods, namely electrochemical impedance spectroscopy (EIS) and Fourier transform infrared (FTIR) spectroscopy, were used to observe the changes of the three laccase-based biosensors. In the case of CNT-GNP-Lac/SPCE, the surface morphology was studied by SEM.

VIII.2.1. EIS Study for CNT-Lac/SPCE, GNP-Lac/SPCE and CNT-GNP-Lac/SPCE

To clarify the differences in the electrochemical performance of modified electrodes, EIS was used as a technique for electrochemical characterization of their surfaces as well as the charge transfer resistance for each immobilization, which indicates the interaction of the enzyme with the substrate [95]. The impedance (Z) represents the total resistance that the circuit offers to the alternating current flow at a given frequency [96].

Figure VIII.1 shows impedance spectra in the form of Nyquist plots in which the imaginary impedance (Zim) is plotted against the real impedance (Zre) as a function of frequency for 1 mM $[Fe(CN)_6]_3$ − and 1 mM $[Fe(CN)_6]_4$− at all unmodified electrodes including CNT/SPCE,

GNP/SPCE, CNT-GNP/SPCE and Lac-modified electrodes including CNT-Lac/SPCE, GNP-Lac/SPCE and CNT-GNP-Lac/SPCE in 10^{-1} M KCl.

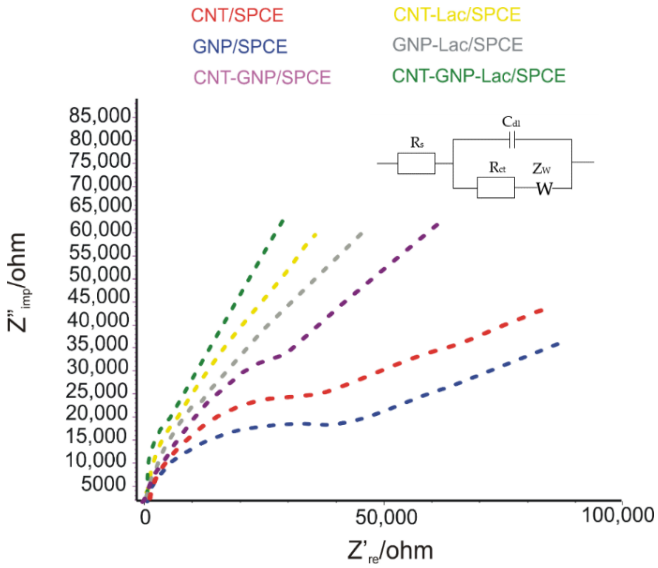


Figure VIII.1. *Nyquist plots for 1 mM $[Fe(CN)_6]_3$ − and 1 mM $[Fe(CN)_6]_4$− obtained at CNT-Lac/SPCE, GNP-Lac/SPCE and CNT-GNP-Lac/SPCE, in 10^{-1} M KCl, open circuit mode, 10 mV amplitude and frequency range of 100 MHz–100 kHz.*

It can be appreciated that the adsorption of Lac on the surface of CNT-GNP-Lac/SPCE is directly related to the decrease of the semi-circle and the increased electron transfer (880 Ohm) due to the coating of the electrode surface, confirming the presence of a new conductive layer, thus demonstrating the GNPs present in the CNT film enhance the electron transfer between the reactant and the electrode surface [97].

VIII.2.3. Morphological Characterization through SEM

Figure VIII.2 shows the SEM image highlighting the surface morphology of the composite nanofilm containing CNTs, GNPs and the enzyme Lac.

Figure VIII.2 *Scanning electron micrograph of the active surface of CNT-GNP-Lac/SPCE*

VIII.3. The Influence of pH on the Performance of Biosensors

In order to establish an optimal pH value at which further determinations will be carried out in this study, the electrochemical behavior of the three biosensors in 10^{-1} M acetate buffer

with different pH values (3.0, 4.0, 5.0, 6.0) was evaluated, at a scan rate of 0.1 $V \cdot s^{-1}$. When CNT-Lac/SPCE, GNP-Lac/SPCE and CNT-GNP-Lac/SPCE were immersed in a 10^{-1} M acetate buffer at various pH values, the cyclic voltammograms showed, in all cases, two peaks: an anodic one of low intensity and a cathodic one that is more obvious. It can be clearly observed that for a pH higher than 6.5, the response decreases dramatically, and a maximum response is reached at about pH 5.2. The peaks are related to the electrochemical reducing process of Lac on the surface of the modified electrodes.

VIII.4. Electrochemical Behavior of Electrodes in Catechin Solution

The qualitative and then quantitative determination of catechin was carried out by CV and DPV, these methods being useful for the interpretation of processes occurring at the electrode surface. In the case of CV, the scan rate used was 0.1 $V \cdot s^{-1}$.

Figure VIII.3 shows the cyclic voltammograms of 10^{-3} M catechin at the three electrodes in 10^{-1} M acetate buffer (pH 5.2). In order to obtain a stable sensor response, three cycles in the optimized potential range (−0.4 V to 0.7 V) were required.

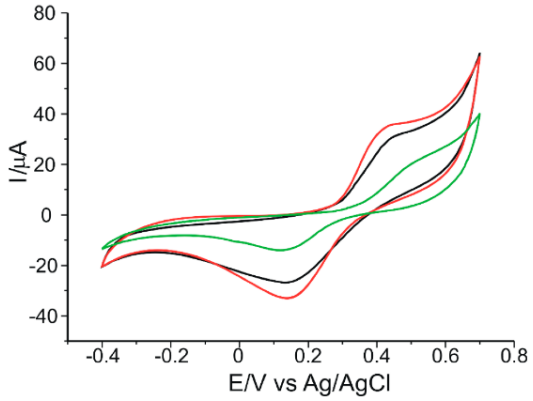


Figura VIII.3. *CVs of 10^{-3} M catechin at a CNT-Lac/SPCE (black trace), a GNP-Lac/SCPE (green trace) and a CNT-GNP-Lac/SPCE (red trace) in 10^{-1} M acetate buffer (pH 5.2). Scan rate: 0.1 $V \cdot s^{-1}$.*

Figure VIII.3 shows that the oxidation and reduction drop intensities are higher for CNT-GNP-Lac/SPCE, which can be attributed to the synergistic effect of the association of CNTs with GNPs.

The electrochemical parameters obtained from the cyclic voltammograms of 10^{-3} M catechin at the three modified biosensors are shown in Table VIII.1.

Table VIII.1. *The values of the parameters obtained from the cyclic voltammograms of 10^{-3} M catechin at electrodes in 10^{-1} M acetate buffer (pH 5.2) supporting electrolyte.*

Sensor	Epa(V)	Ipa(µA)	Epc(V)	Ipc(µA)	Ipc/Ipa	ΔE(V)	$E_{1/2}$
CNT-Lac/SPCE	0,467	29,76	0,136	-26,90	0,90	0,331	0,301
GNP-Lac/SPCE	0,527	19,70	0,130	-14,08	0,71	0,397	0,328
CNT-GNP-Lac/SPCE	0,464	34,20	0,150	-34,58	1,01	0,314	0,307

The Ipc/Ipa ratio is close to the ideal value of 1 in all three cases, the closest value being obtained in the case of CNT-GNP-Lac/SPCE (1.01). Taking into account this value, but also the fact that for this modified sensor the difference between anodic and cathodic peak potentials (ΔE) is nearest to 29.5 mV, it can be stated that CNT-GNP-Lac/SPCE has the highest degree of reversibility. Also, for this sensor the highest currents were obtained, followed by CNT-Lac/SPCE and then GNP-Lac/SPCE. From these results it can be concluded that the highest sensitivity for catechin detection was obtained for CNT-GNP-Lac/SPCE.

VIII.5. Calibration Curve

The calibration curve for the concentration range of 0.1 μM–10.50 μM catechin obtained through DPV method for CNT-GNP-Lac/SPCE is shown in Figure VIII.4.

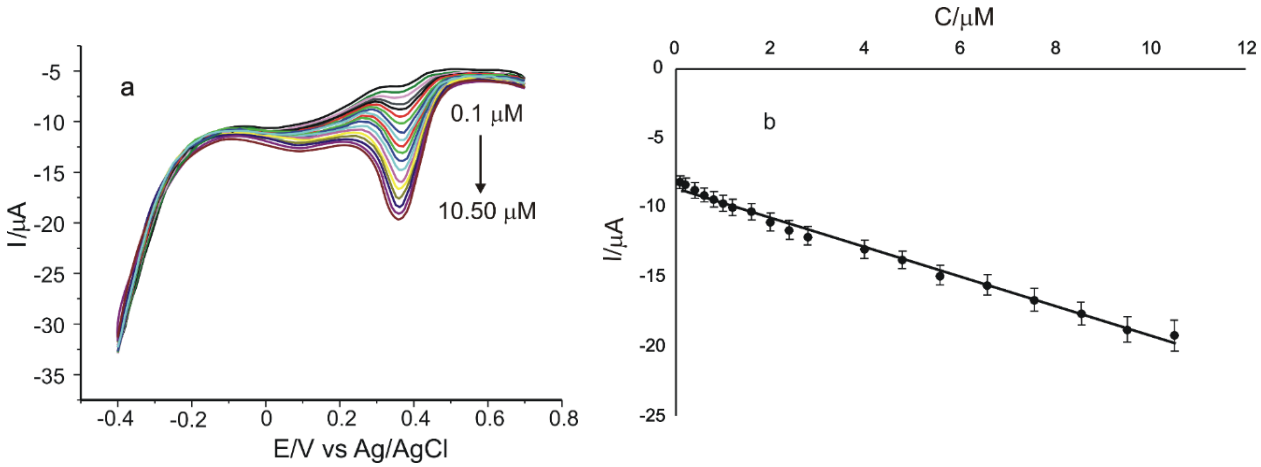


Figura VIII.4. *DPVs recorded for CNT-GNP-Lac/SPCE in the concentration range of 0.1–10.50 μM catechin (a); linear fitting within the range of 0.1–10.50 μM for CNT-GNP-Lac/SCPE (b). The potential range used was −0.4 to 0.7 V, the pulse height was 0.10 V, the pulse width 0.5 s and the scan increment 0.01 V.*

LOD and LOQ values obtained by the DPV method were calculated with all three modified sensors and the results are presented in Table VIII. 2

Tabel VIII.2. *Equation of linear dependence between Ipc and c, R^2 (n = 19), LOD and LOQ for catechin at a CNT-Lac/CE and CNT-GNP-Lac/SPCE based on DPV detection.*

Electrode	Equation	R^2	LOD (M)	LOQ (M)
CNT-Lac/SPCE	Ipc= -1,6068x – 16,228	0,9909	$5,58 \times 10^{-8}$	$1,86 \times 10^{-7}$
GNP-Lac/SPCE	Ipc= -0,9171x – 9,6965	0,9904	$1,29 \times 10^{-7}$	$4,30 \times 10^{-7}$
CNT-GNP-Lac/SPCE	Ipc= -1,0614x – 8,6305	0,9928	$4,89 \times 10^{-8}$	$1,63 \times 10^{-7}$

Due to the synergistic effect of the association of GNPs with CNTs [98], nteraction with catechin is favored, with improved selectivity and sensitivity of the CNT-GNP-Lac/SPE biosensor, which shows better performances than the other two biosensors.

VIII.5.1. Enzyme Kinetics: Calculation of Maximum Reaction Rate and Michaelis–Menten Constant

To evaluate the characteristics of an enzyme in solution, the Michaelis–Menten model is the most widely used, where reaction rates are measured as a function of enzyme-like substrate concentration [99].

The apparent Michaelis–Menten constant (K_M^{app}), an indication of both enzyme affinity and enzyme substrate kinetic constants, is determined from the electrochemical Lineweaver–Burk form of the Michaelis–Menten equation.

The values obtained for the three biosensors are shown in Table VIII.3.

Table VIII.3. *Characteristic parameters obtained with the three biosensors*

Biosensor	I_{max}/µA	K_M^{app}/µM
CNT-Lac/SPCE	-12,61	0,281±0,007
GNP-Lac/SPCE	-14,10	0,299±0,009
CNT-GNP-Lac/SPCE	-23,47	0,269±0,004

It is found that the K_M^{app} values are close for the three biosensors but significantly different at a 95% confidence level. This fact is related to the role of the immobilization matrix in the biocatalytic properties of the enzyme. The lowest value was obtained for CNT-GNP-Lac/SPCE. This suggests that the affinity of the laccase to the substrate is stronger for this biosensor, giving it higher sensitivity [100].

VIII.6. Quantitative Determination of Catechin in Nutraceuticals

In order to validate the biosensor in the catechin analysis from real-life samples, three different products from different manufacturers and containing catechin in different concentrations were selected and analyzed: Green Tea Adams Vision, Green Tea Extract Bio Synergie, Green Tea Extract Zenyth. These products were quantitatively analyzed using the DPV electrochemical method. The aim of this analysis was to compare the results of the experiment and the values indicated by the manufacturers on the label of the nutraceutical products analyzed.

Results obtained are reported as means of three replicates, being expressed in mg catechin per capsule (Table VIII.4).

Table VIII.4. *The results obtained by the CNT-GNP-Lac/SPCE biosensor by interpolation in the calibration plot using DPV as voltammetric method, regarding the amount of catechin in the selected nutraceuticals, compared to those mentioned by the manufacturer on the label.*

Nutraceuticals	Catechin Content Specified by Manufacturer (mg/Capsule)	The Amount of Catechin Determined by DPV Method (mg/Capsule)
Green Tea Adams Vision	-	31,9±1,32
Green Tea Extract Bio Synergie	15	16,8±0,91
Green Tea Extract Zenyth	200	203,4±4,07

As can be seen in Table VIII.4, the results obtained using the DPV method as well as those provided by the producers are similar. The paired t-test assuming equal variances have shown that at 95% confidence level there are no significant differences between the means, which demonstrates the accuracy of the catechin quantification method presented in this study. Therefore, the biosensor could be successfully applied in laboratory practice in the quality control of pharmaceutical products containing catechin.

VIII.7. Evaluation of Antioxidant Activity by Spectrophotometric Methods

We have studied the ability of catechin to neutralize free radicals using DPPH, ABTS and galvinoxyl assays.

In the case of the three nutraceuticals studied, the results for the percentage inhibition values are shown in Table VIII.5.

Table VIII.5. *Antioxidant activity of the studied nutraceutical formulations*

Nuctraceutical Product	% Inhibition-DPPH	% Inhibition-Galvinoxyl	% Inhibition-ABTS
Green Tea Adams Vision	19,36	19,25	31,07
Green Tea Extract Bio Synergie	18,34	18,99	31,22
Green Tea Extract Zenyth	70,63	70,65	95,70

It is noted that the results of the DPPH and galvinoxyl assays differ from those of the ABTS assay. This is probably related to the different type of reagents used and relative poor selectivity of ABTS in the reaction with hydrogen-atom donors (i.e., catechin) comparing with DPPH or galvinoxyl [90].

VIII.8. Determination of Antioxidant Activity by Electrochemical Methods

By means of voltammetric methods, the main electrochemical parameters (Ep and Ip) can be obtained, thus giving insights into the presumed antioxidant activity of each sample analyzed..

VIII.8.1. CV–Anodic Area

According to the literature, the area under the anodic peak curve (Sa) can express the total reducing power of complex mixtures of antioxidant compounds, such as green tea extracts. Each anodic peak reflects a component or combination of components that donate electrons at approximately the same potential. Therefore, the Sa of CVs of all three nutraceuticals recorded at a CNT-GNP-Lac/SPE can be correlated with the antioxidant activity of the respective products. Figure VIII.5 illustrates the CVs of the three products studied, in the potential range −0.4 and 0.7 V, recorded at a scan rate of 0.1 $V \cdot s^{-1}$, using the CNT-GNP-Lac/SPCE biosensor. Sa corresponds to the charge used in the experiment from the potential of 0.08 V to 0.4 V (Q400) and is used as a measure of the antioxidant content of the products.

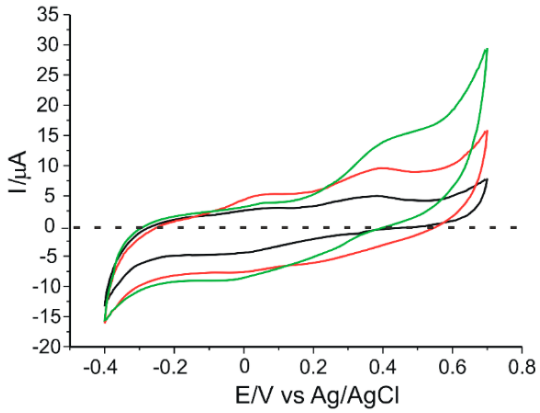


Figura VIII.5. *CVs of 9 mg product/50 mL 10^{-1} M ABS where the product is Green Tea Adams Vision (red trace), Green Tea Extract Bio Synergie (black trace) and Green Tea Extract Zenyth (green trace) at a CNT-GNPLac/SPCE. Scan rate: 0.1 V·s^{-1}.*

Table VIII.6 shows the values of the electrochemical parameters obtained from the cyclic voltammograms shown in Figure 5 and the Q400 values (expressed in µC) for the three pharmaceuticals studied. It is noted that the highest Q400 value was obtained for the Green Tea Extract Zenyth product, which can be attributed to the high amount of antioxidant compound, namely catechin, contained per capsule, compared to the other two products, as demonstrated earlier in this study by spectrophotometric methods.

Table VIII.6. *The values of the parameters obtained from the cyclic voltammograms of all three nutraceutical products recorded with CNT-GNP-Lac/SPCE biosensor at a scan rate of 0.1 V·s^{-1}.*

Nutraceutical Product	Epa (V)	Ipa (µA)	Q400 (µC)
Green Tea Adams Vision	0,375	8,20	2,27
Green Tea Extract Bio Synergie	0,388	4,85	0,90
Green Tea Extract Zenyth	0,351	12,42	6,18

VIII.8.2. DPV-Electrochemical Index (EI)

EI has been defined as a screening method meant to determine the total concentration of antioxidant compounds in different samples and can be obtained using electrochemical techniques, e.g., CV and DPV, taking into account the type of sample to be analyzed, Ep and Ip.

To determine the EI of the three nutraceuticals studied, the values of the intensities and potentials obtained by the electrochemical DPV method were taken into account. At the same time, the EI was also determined for pure catechin in order to compare the results These are presented in Table VIII.7.

Table VIII.7. *Results of the electrochemical index of catechin and of the three nutraceuticals studied.*

	Catechin	Green Tea Adams Vision	Green Tea Extract Bio Synergie	Green Tea Extract Zenyth
EI (µA/mV)	0,76	0,72	0,71	0,78

It appears from the table that the highest value of EI was obtained in the case of Green Tea Extract Zenyth, approximately equal to that of the pure substance at a concentration of 10^{-3} M. At the same time, for the other two products, EI value was close in value and can be compared with EI value of catechin.

VIII.9. Conclusions

In the present work, three Lac-based biosensors, namely CNT-Lac/SPCE, GNP-Lac/SPCE and CNT-GNP-Lac/SPCE, were developed and characterized, with the CNT-GNP-Lac/SPCE showing the best analytical performance with an LOD of 4.89×10^{-8} M and a sensitivity of 8.63 mA M^{-1}. This can be attributed to the association of CNT with GNP, which increased the sensitivity of the biosensor significantly due to higher electroactivity as well as easier electron transport to the electrode surface.

This paper also brings together two types of methods, chemical (DPPH, galvinoxyl and ABTS) and electrochemical (CV and DPV), to characterize the antioxidant activity of catechin and of the three nutraceuticals studied. Thus, by means of the DPV voltammetric method it was possible to determine the electrochemical index of the pure compound and of the nutraceutical products, and by means of CV, Sa (correlated with the antioxidant activity) was evaluated. Both methods showed that the highest antioxidant activity was obtained in the case of Green Tea Extract Zenyth, being comparable to that of the pure compound, at a concentration level of 10^{-3} M.

CHAPTER IX. VOLTAMETRICE DETECTION OF ROSMARINIC ACID IN COSMETICS WITH A NEW ELECTROCHEMICAL SENSOR BASED ON GRAPHENE OXIDE MODIFIED WITH OCTAPEPTIDE

The objective of this study was to manufacture a new sensor, using a zwitterionic peptide, fixed by means of a cross-linking agent on the surface of a SPCE modified with a composite go film, with applicability for the determination and quantification of rosmarinic acid in cosmetic products with complex composition. The aim was also to optimize the experimental conditions in terms of the pH of the solution and the quantities of peptide used to modify the surface of the sensor, so that the analytical performance of the new sensor is optimal for the detection of rosmarinic acid. The FT-IR method was used to validate the results obtained by applying the voltametric method.

IX.1. Sensor Preparation

IX.1.1. Zwitterionic peptide – description

In recent years, peptides have been proposed as promising and versatile elements for the design and development of sensors [101], [102]. Peptides can be easily obtained by chemical synthesis methods, avoiding the need for in vivo, laborious procedures, as with antibodies [103]. The benefits of using these compounds consist mainly in their stability and selectivity over a target analyte, and can be modified with specific functional groupings, thus making them suitable for the development of new bio-recognition platforms.

IX.1.2. Sensor preparation by immobilizing the zwitterionic peptide

The GO/SPCE electrode was used as support to prepare the sensor. Briefly, a stock solution of peptide was prepared, and 20 µL of this solution was drop-casted onto the working

electrode, sequentially, in four steps (5 µL in each step) and then allowed to dry for about two hours between each step. Glutaraldehyde 2% (v/v) was used as an immobilisation agent.

IX.2. Electrode Characterization

In order to highlight the changes in the commercial GO-based sensor, its active surface was analyzed using two methods: FTIR spectrometric method and SEM.

IX.2.1 FTIR Spectrometric Method

The first method used to characterize the modified sensor was the infrared spectrometric method. The results are shown in Figure IX.1

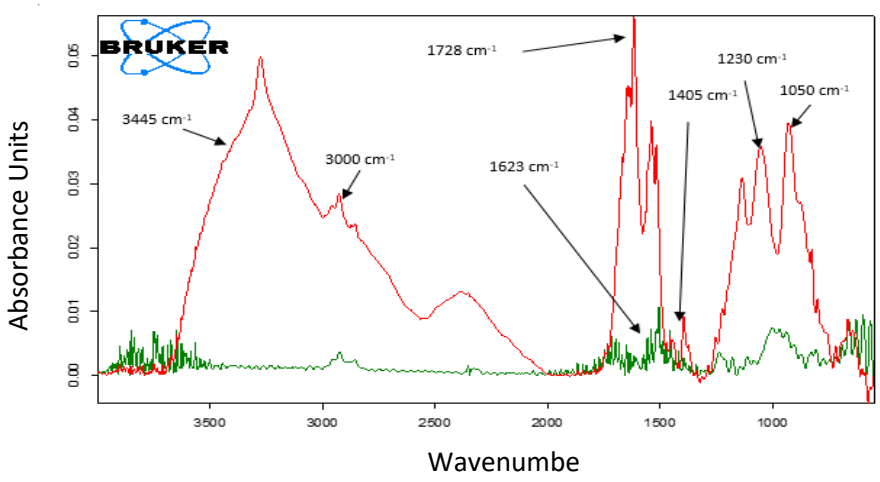


Figure IX.1. *FTIR spectra for GO/SPCE (green line) and GO-Peptide/SPCE (red line).*

Several peaks representative of the presence of GO can be noticed in the wavenumber range 1050–1728 cm^{-1} [104]. he absorption at about 1623 cm^{-1} is associated with the stretching vibration of the benzene ring C=C [105]. At the same time, the band at 1728 cm^{-1} occurs due to the C=O [106], while the band at 1405 cm−1 can be attributed to the C–O [107], which includes both the 1230 cm^{-1} epoxy C–O [108], and the 1050 cm−1 alkoxy C–O [109], located at the edges of the graphene oxide.

IX.2.2. Morphological Characterization Using SEM

Figure IX.2 depicts the SEM images, emphasizing the surface morphology of the composite nanofilm containing GO and peptide

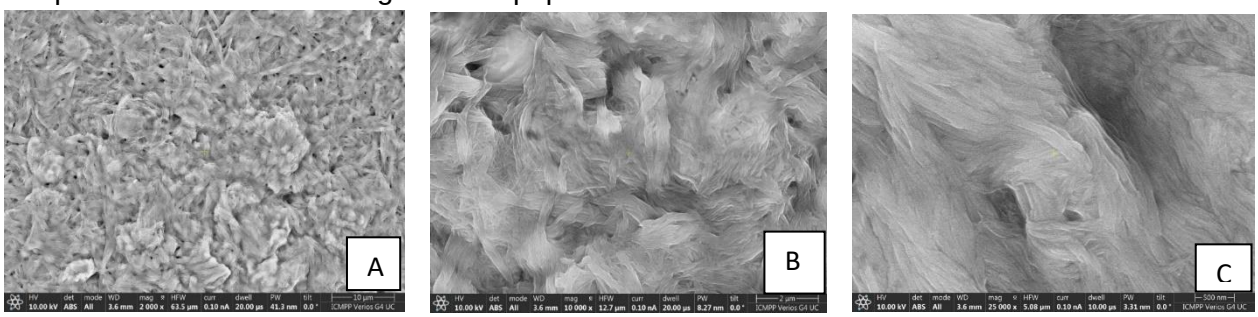


Figure IX.2. *Scanning electron micrograph at different magnifications representing the active surface of GO-Peptide/SPCE: (A) 2000 times magnification; (B) 10,000 times magnification; (C) 25,000 times magnification.*

IX.3. Electrochemical Properties of GO-Peptide/SPCE in $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ Solution

The next step consisted in analyzing the electrochemical response of both the modified and unmodified sensor in a solution containing 10^{-3} M $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ dissolved in 0.1 M PBS, pH = 6.5, recording cyclic voltammograms from −0.4 to 1.0 V.

Figure IX.3 describes the stable sensor signals. It can be seen that, for the GO/SPCE, there is a pair of redox peaks, one anodic and one cathodic.

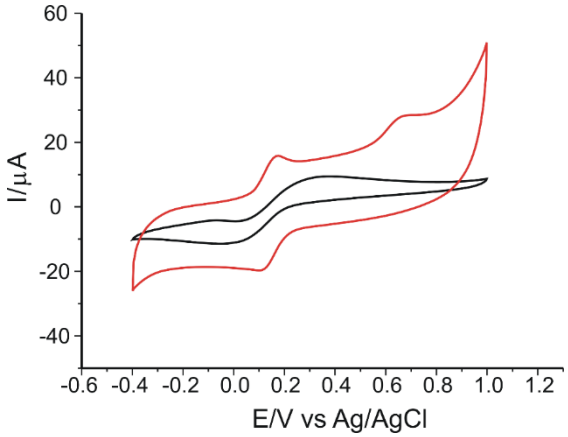


Figure IX.3. *Cyclic voltammograms of GO/SPCE (black line) and GO-Peptide/SPCE (red line) immersed in 10^{-3} M $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$-0.1 M PBS solution, at the scan rate of 0.05 $V \cdot s^{-1}$.*

Table IX.1 includes the main parameters obtained from cyclic voltammograms for peaks related to the electrochemical processes of the $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$

Table IX.1. *Electrochemical parameters obtained from cyclic voltammograms in $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 10^{-3} M – PBS 10^{-1}M solution*

Sensor	Ipa(μA)	Ipc(μA)	Ipc/Ipa	Epa (V)	Epc(V)	$E_{1/2}$ (V)	ΔEp (V)
GO/SPCE	9,34	-11,36	1,21	0,361	-0,02	0,190	0,341
GO-Peptide/SPCE	15,58	-19,42	1,24	0,286	0,173	0,229	0,113

As can be seen from Table IX.1, both sensors have an Ipc/Ipa ratio that exceeds the ideal value 1. This shows that the process of oxidation-reduction is quasi-reversible [110].

To study the kinetics of the redox process, cyclic voltammograms were recorded for GO/SPCE and GO-Peptide/SPCE using 10^{-3} M $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$-0.1 M PBS solution at pH = 6.5 with scan rates ranging from 0.05 to 0.5 $V \cdot s^{-1}$. The results obtained are presented in Figure IX.4.

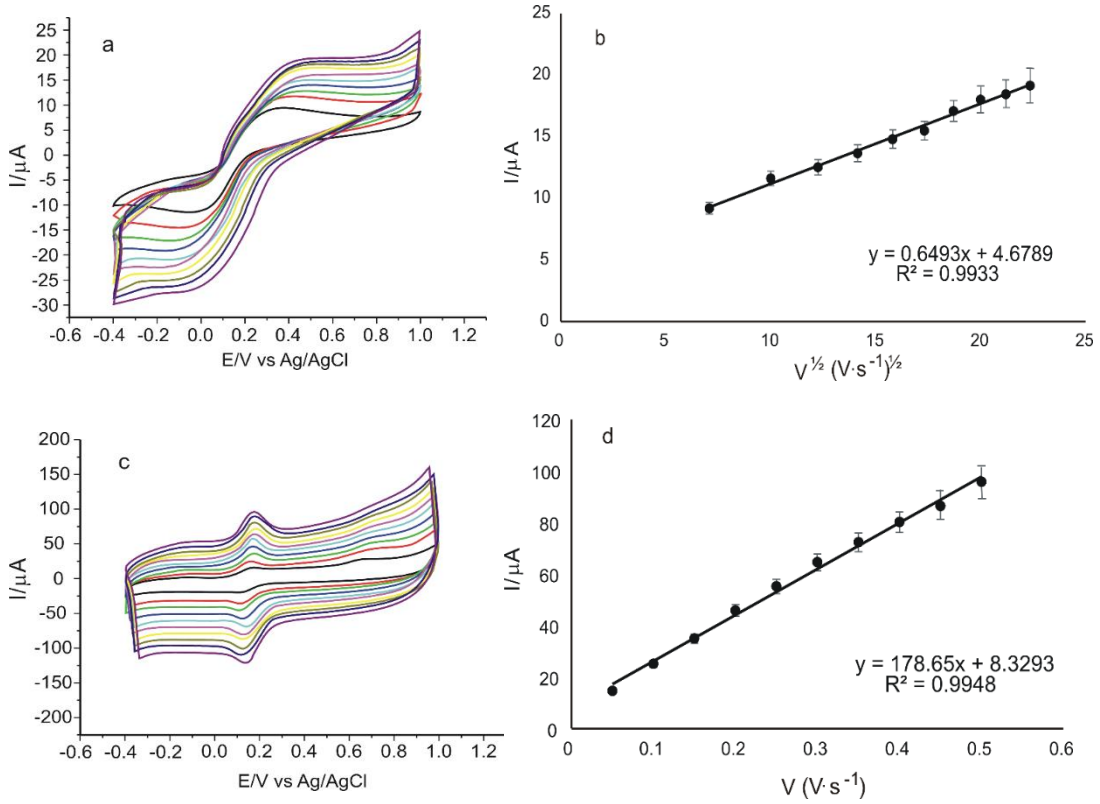


Figure IX.4. *Cyclic voltammograms of GO/SPCE (a) and GO-Peptide/SPCE (c) immersed in 10^{-3} M $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$-0.1 M PBS solution at pH = 6.5 recorded at scan rates between 0.05 and 0.5 $V \cdot s^{-1}$. Linear dependence of Ipa and square root of scan rate in the case of GO/SPCE (b) and linear dependence of Ipa and scan rate in the case of GO-Peptide/SPCE (d).*

For GO/SPCE, a linear dependence is observed between Ipa and the square root of the scan rate, as depicted in Figure IX.8b. This linear dependence suggests that the oxidation reduction of potassium ferrocyanide is controlled by the diffusion of the electroactive species, this being the determining stage of the electrochemical process [111]. In the case of GO-Peptide/SPCE, there is a linear dependence between Ipa and scan rate, which suggests that the electrochemical process at the electrode surface is controlled by the adsorption of the electroactive species [112] (Figure IX.8d)). This change in the determining factor of the oxide-reduction process in the case of GO-Peptide/SPCE is due to the modification of the sensor with the peptide, which has a major effect on the reaction kinetics at the electrode surface and provides a suitable environment for a sensitive layer–analyte interaction and rapid electron transfer [113].

IX.4. Electrochemical Responses of Sensors in Rosmarinic Acid Solution

Electrochemical behavior of GO/SPCE and GO-Peptide/SPCE in rosmarinic acid solution was investigated using CV.

În Figure IX.5 shows the response of the two electrodes, GO/SPCE and GO-Peptide/SPCE, when immersed in a solution of 10^{-3} M rosmarinic acid-0.1 M PBS (pH = 6.5). Three cycles in the optimized potential range (−0.4 V to 1.0 V) were required to achieve a stable sensor response. The cyclic voltammograms shown in Figure IX.5 were achieved after stabilizing the signals.

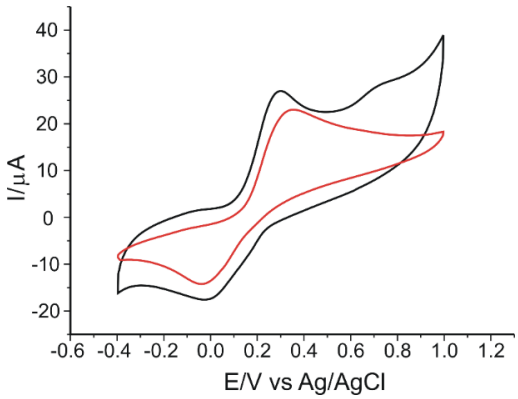


Figure IX.5. *Cyclic voltammograms of GO/SPCE (red line) and GO-Peptide/SPCE (black line) immersed in 10^{-3} M rosmarinic acid-0.1 M PBS solution (pH 6.5). Scan rate: 0.05 V·s^{-1}.*

In both cases, a pair of well-defined peaks having different intensities is shown, namely an anodic peak, representing the oxidation of rosmarinic acid to the corresponding o-quinone, and a cathodic peak, corresponding to the electrochemical reduction back to rosmarinic acid. In the case of the peptide-modified sensor, a shift in the oxidation potential towards more negative values and an increase in Ipa was observed, demonstrating that the peptide, in combination with GO, confers an improved conductivity to the sensor and a thermodynamically favored antioxidant activity to the compound. In the case of GO-Peptide/SPCE, the appearance of the second anodic peak at the potential value of 0.72 V due to the presence of peptide on the SPCE surface is observed, as previously demonstrated in the experimental parameter optimization studies.

IX.5. Calibration curve and determination of detection limit

The variation of the sensor response depending on the concentration of the rosmarinic acid solution analyzed is shown in Figure IX.6a. It can be observed that the anodic and cathodic peak intensities increase with the increase in concentration. In Figure IX.6b the dependence between Ipa and the rosmarinic acid concentration in the range of 0.1–3.20 µM can be observed, in the case of using GO-Peptide/SPCE.

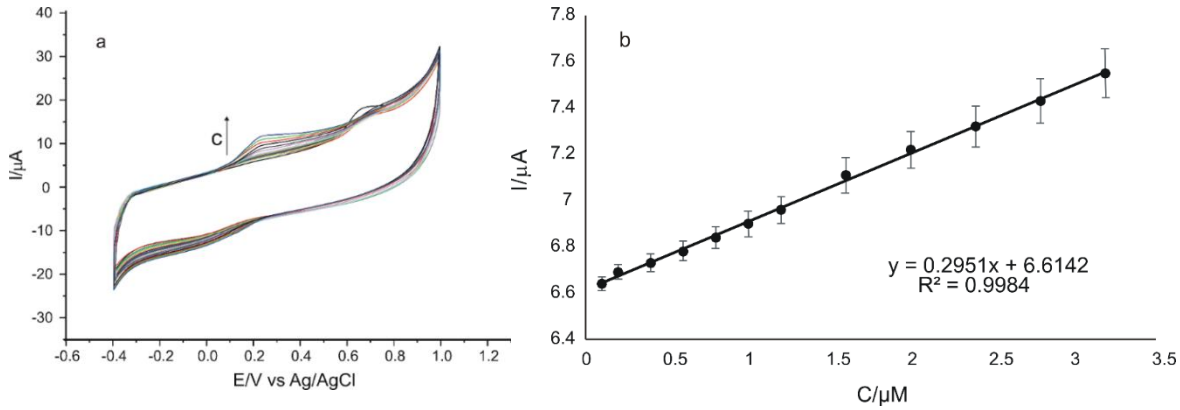


Figure IX.6. *Cyclic voltammograms recorded for GO-Peptide/SPCE with the concentration between 0.1 and 3.20 µM rosmarinic acid (a); Linear dependence between Ipa and rosmarinic acid concentration in the range 0.1–3.20 µM (b).*

Table IX.2 shows the LOD and LOQ values, calculated according to the equations described in previous studies, obtained with GO-Peptide/SPCE.

Table IX.2*. Data obtained from the calibration curves for GO-Peptide/SPCE during rosmarinic acid detection.*

Electrode	Equation	R^2	LOD ($\mu mol \cdot L^{-1}$)	LOQ ($\mu mol \cdot L^{-1}$)
GO-Peptide/SPCE	Ipc= 0,2951c + 6,6142	0,9984	0,0966	0,322

Due to the presence of the peptide, which favors the interaction with rosmarinic acid and gives the sensor sensitivity, it outperforms other sensors used to determine rosmarinic acid [114], [115], [39], [116].

IX.6. Quantitative Determination of Rosmarinic Acid in Cosmetic Products

aking into consideration its superior characteristics, the GO-Peptide/SPCE sensor was used in subsequent analysis to quantify rosmarinic acid in three cosmetic products, namely Apiterra anti-aging cream for sensitive skin, Sabio soothing and repairing balm with herbal extract, and Vivanatura Mattifying Moisturizing Cream for mixed-type skin. Two methods were used for the analysis of the three cosmetic products, namely CV (the method previously described in this work) and FTIR (Fourier transform infrared spectroscopy) (the standard method) [89].

Three different well-established amounts of each product were used to obtain the solutions to be tested: 0.1 g, 0.2 g, and 0.3 g. Applying CV, measurements were recorded from −0.4 V to 1.0 V, at a scan rate of 0.05 $V \cdot s^{-1}$.

On the basis of the anodic peak current, the amount of cosmetic product taken in the analysis, and the equation of the calibration line, the concentrations of rosmarinic acid in cosmetics were calculated, and the results are included in Table IX.3. All the analyses were carried out in triplicate.

Table IX.3. *Rosmarinic acid concentrations obtained from the two analysis methods used in this study.*

Cosmetic product	c% Rosmarinic Acid Voltammetric Method	c% Rosmarinic Acid FTIR Spectroscopy
Apiterra	0,288±0,03	0,321±0,05
Sabio	1,042±0,08	1,061±0,09
Vivanatura	0,081±0,01	0,076±0,01

Analysis of variance showed that there is no significant difference between the results obtained with both methods at a 99% confidence level, which demonstrates that the voltammetric method using the new GO-Peptide/SPCE sensor is useful for the quantification of rosmarinic acid with adequate precision and selectivity, this representing the present study's novelty.

IX.7. Conclusions

A novel sensor was developed by immobilizing a zwitterionic peptide, containing the H2N-D-V-C-Y-Y-A-S-R-COOH sequence, on the surface of an SPE modified with a conductive material with excellent properties, namely a GO composite film.

Using as a method of detection of CV, very good results could be obtained, with applicability in laboratory practice. The concentration range in which GO-Peptide/SPCE was tested ranged from 0.1 μm to 3.20 μm with a detection limit of $9.66 \cdot 10^{-8}$ M, a low value compared to other literature-reported devices developed for the determination of the same compound, namely rosmarinic acid.

CHAPTER X. QUANTITATIVE DETERMINATION AND EVALUATION OF THE ANTIOXIDANT ACTIVITY OF VERBASCOSIDE IN OLIVE OIL BY MEANS OF A NEW ELECTROCHEMICAL SENSOR BASED ON GRAPHENE OXIDE, MODIFIED WITH PENTAPEPTIDE

In this study, a direct electrochemical method is described, capable of assessing and quantifying verbascoside content in different samples of extra virgin olive oils, with minimal sample preparation, using a SPCE, modified with a composite go film, on whose surface a pentapeptide was immobilized (Sequence NH_2-FESNF-CO-NH_2) via glutaraldehyde as a cross-linking agent. The validation of the electroanalytical method was carried out using the standard addition method, achieving very good results.

X.1. Obtaining the Chemically Modified Sensor

In this study, to obtain the chemically modified sensor, we used pentapeptide containing the sequence NH_2-FESNF-CO-NH_2, where F is phenylalanine, E is glutamic acid, S is serine, and N is asparagine.

The same steps described in the previous study were followed for the preparation of the SPCE/GO-Pentapeptide sensor.

X.2. Electrode Characterisation

In order to observe the changes to the commercial GO-based sensor, its active surface was analyzed using two methods, namely the FT-IR spectrometric method, highlighting the absorption bands corresponding to the presence of functional groups of pentapeptide and SEM, highlighting the three-dimensional arrangements made of continuous fibers with good homogeneity (figure X.1).

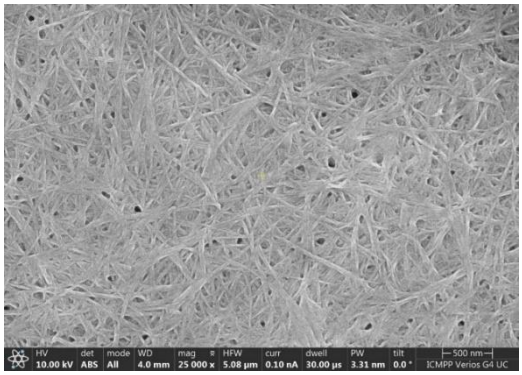


Figure X.1. *SEM image representing the active surface of SPCE/GO-Pentapeptide*

X.3. Electrochemical Sensor Responses in Verbascoside Solution

The next step of the present study was to analyze the behavior of both the modified and the unmodified sensor in verbascoside solution using CV.

Figure X.2 shows, by comparison, the response of the two sensors when immersed in a solution of verbascoside 10^{-4} M - PBS 0.1 M (pH = 6.5). In order to obtain a stable sensor response, three successive cycles in the optimized potential range (−0.4 V to 1.0 V) were required. The CVs shown in Figure X.2 are obtained after the stabilization of the signals.

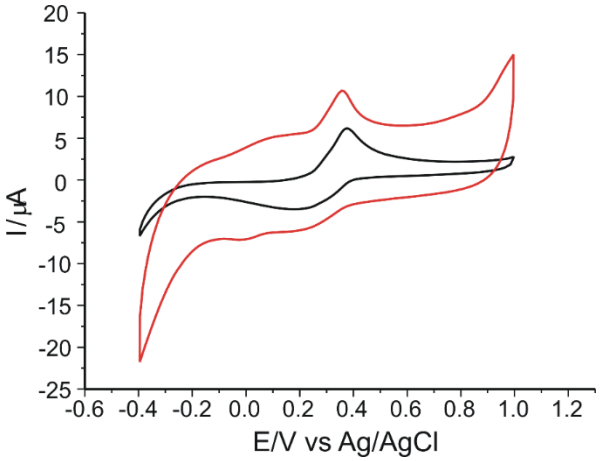


Figure X.2. *CVs of* SPCE/GO *(black line) and SPCE/GO-Pentapeptide (red line) immersed in 10^{-4} M verbascoside–0.1 M PBS solution (pH 6.5). Scan rate: 0.05 V·s^{-1}.*

Regarding the behavior of the peptide-modified sensor upon immersion in verbascoside solution, it is different from the unmodified sensor, which is evident in the corresponding voltammogram (red line) in Figure X.2. Thus, the oxidation of verbascoside occurs in two steps, showing two anodic and two cathodic peaks of different intensities and potentials.

The electrochemical parameters achieved from the CVs of the two sensors immersed in 10^{-4} M verbascoside solution are presented in Table X.1.

Table X.1. *The values of the parameters obtained from the CVs of the two sensors immersed in 10^{-4} M verbascoside - 0.1 M PBS solution of pH 6.5.*

Sensor	Epa_1 (V)	Epa_2 (V)	Ipa_1 (μA)	Ipa_2 (μA)	Epc_1 (V)	Epc_2 (V)	Ipc_1 (μA)	Ipc_2 (μA)
SPCE/GO	0,38	-	6,05	-	0,21	-	-3,46	-
SPCE/GO-Pentapeptide	0,08	0,35	4,79	10,74	0,01	0,22	-7,20	-5,22

The reduced value of the first anodic peak potential in the case of SPCE/GPHOX-Pentapeptide indicates a lower activation energy of the oxidation process, being influenced by the adsorption of verbascoside on pentapeptide predominantly through hydrogen bonds [117] and through π–π stacking interactions between pentapeptide and verbascoside aromatic rings [118]. Therefore, in the case of this sensor, the electron transfer occurring at the active surface is faster compared to the unmodified sensor [119].

The next step was the study of the influence of scan rate on the voltammetric responses of the two sensors in a solution of 10^{-4} M verbascoside - 0.1 M PBS, pH = 6.5, applying scan

rates in the range 0.05–0.5 $V \cdot s^{-1}$ (Figure X.3). Since the anodic peaks are higher and better defined compared to the catodic ones, the dependence of Ipa on the scan rate or square root of the scan rate will be studied.

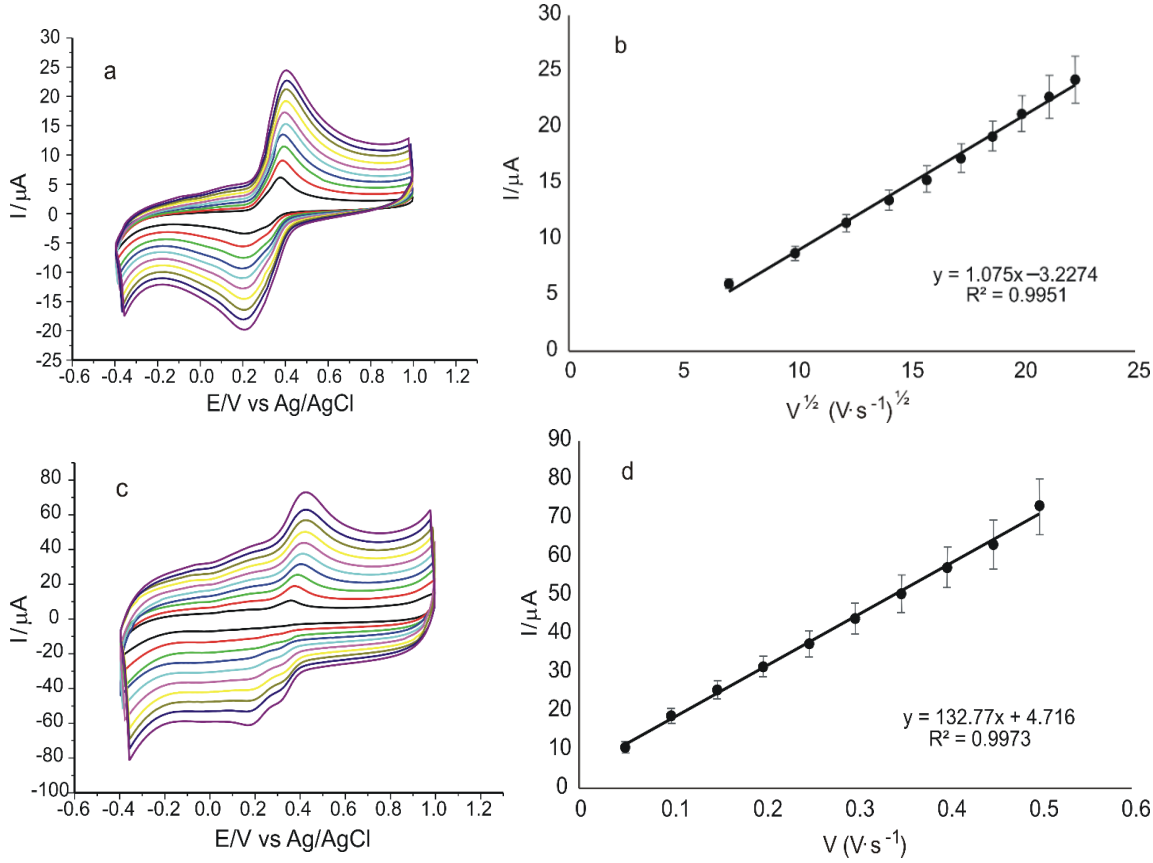


Figure X.3. *CVs of SPCE/GO (a) and SPCE/GO-Pentapeptide (c) immersed in 10^{-4} M verbascoside - 0.1 M PBS at pH = 6.5 recorded at scan rates between 0.05 and 0.5 $V \cdot s^{-1}$. Linear dependence between Ipa and square root of scan rate in the case of SPCE/GO (b) and linear dependence between Ipa and scan rate in the case of SPCE/GO-Pentapeptide (d).*

By increasing the scan rate, it was obvious that the maximum oxidation potentials for both sensors (Figure X.3a și Figure X.3c) were shifted toward more positive values and the reduction potentials toward more negative values. Moreover, the anodic peak intensities were higher in the modified sensor compared to those obtained with the unmodified sensor, the presence of peptide on the sensor surface significantly improving its sensitivity.

X.4. Development of Calibration Curve

to determine the influence of verbascoside concentration on the SPCE/GO-Pentapeptide response, CVs were recorded in verbascoside solutions of different concentrations obtained by dissolving in 0.1 M PBS solution. The electrochemical sensor responses recorded by CV are shown in Figure X.11a, and in Figure X.11b it can be seen the linear dependence between the Ipa and verbascoside concentration in the linearity range 0.1–10.55 µM when using SPCE/GO-Pentapeptide.

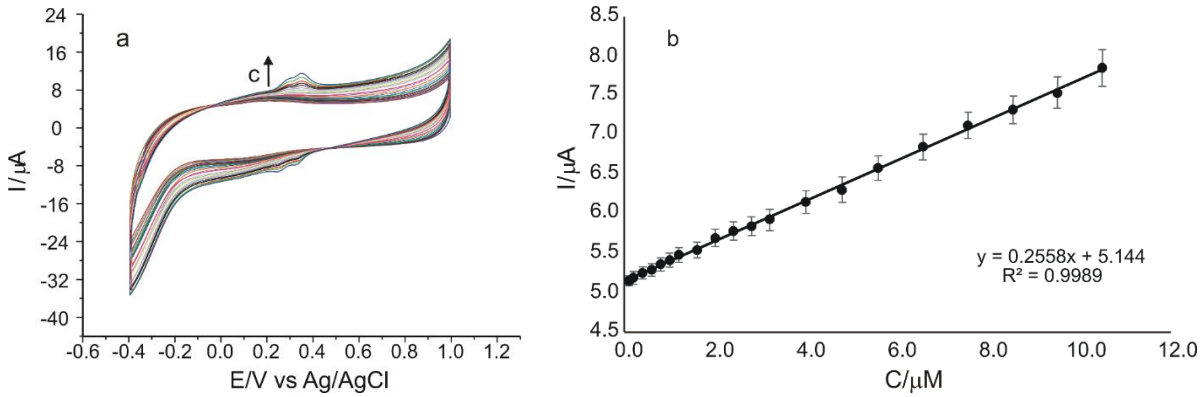


Figure X.4. *CVs recorded for SPCE/GO-Pentapeptide with the concentration between 0.1 µM and 10.55 µM verbascoside (a); Linear dependence between Ipa and verbascoside concentration in the range 0.1–10.55 µM (b).*

As shown in Figure X.4a, a linear relationship was obtained between the intensity of the drops and the verbascoside concentration in the range 0.1-10.55 µm. The calibration right equation calculated the two parameters, LOD and LOQ, using the equations presented in previous studies.

The values obtained for SPCE/GO-Pentapeptide are: LOD = 9.38 x 10^{-8} M and LOQ = 3.12 x 10^{-7} M. Given that these obtained values are similar or lower than those reported in the literature [120],[121] this sensor has been used for the quantitative determination of verbassoside from real samples, i.e. samples of different EVOO.

X.5. Stability, Reproducibility, Repeatability and Interference Studies

The stability of the SPCE/GO-Pentapeptidă was evaluated by performing 30 measurements at regular intervals (one day) for one month using a 10^{-4} M verbascoside solution, using the CV method. Between determinations, the sensor was stored in the refrigerator at 4 °C in a hermetically sealed box. The results showed no significant differences between the anodic currents recorded on different days, with coefficients of variation less than 5%, confirming that the sensor is stable and can be used in electroanalysis.

The reproducibility of the developed method was investigated by preparing five different sensors, using a 10^{-4} M verbascoside solution for performing the test. The calculated RSD value for the Ipa depicted for all five sensors was 3.5%, demonstrating good reproducibility of sensor development.

Tests for repeatability study were performed in a 50 µM verbascoside-0.1 M PBS solution. The value of the coefficient of variation for the anodic peak determined in five consecutive measurements in the same solution did not exceed 2.5%. Between measurements, the SPCE/GO-Pentapeptide was rinsed with 0.1 M PBS solution, pH = 6.5. Therefore, the sensor can be used repeatedly for the determination of verbascoside.

Under optimal experimental conditions, the effect of possible interferents in the form of organic compounds predominantly found in EVOO, such as tyrosol, hydroxytyrosol and oleuropein, on the quantification of verbascoside was evaluated using CV as a detection method. The limit of tolerance was defined as the maximum concentration of interfering compounds that results in a relative error of ±5% for the quantitative determination of

verbascoside. The achieved values show that the peaks related to the presence of our compound of interest do not change significantly upon the addition of interferents.

X.6 Quantitative Determination of Verbascoside in EVOO

To validate the SPCE/GO-Pentapeptide sensor in the analysis of verbascoside from real samples, ten samples of different EVOOs were selected and subjected to analysis using the CV method for determination. Measurements were performed in the potential range between −0.4 V and 1.0 V, applying a scan rate of 0.05 $V \cdot s^{-1}$.

For the calculation of the amount of verbascoside in each sample, the slope of the calibration line obtained by the voltammetric method was used, and the obtained values are shown in Table X.2. All analyses were performed in triplicate.

Table X.2. *Concentrations of verbascoside (n = 3) in commercial EVOO obtained by the voltammetric method.*

EVOO Samples	mg/kg Verbascoside Achieved by CV	RSD (±%)
Pietro Coricelli	1,36	0,02
TopSeller Oil	1,42	0,03
Regina	1,38	0,01
Mazza	1,41	0,03
Olitalia	1,49	0,04
Costa d'Oro Il Grezzo	1,54	0,04
Minerva	1,55	0,06
Costa D'Oro L'extra	1,72	0,08
Monastiri	1,36	0,02
Rivano Olio	1,39	0,03

The values obtained for the 10 samples of EVOO on the concentration of verbascoside are close, the highest value being obtained for Costa D'Oro L'extra (Italy).

The precision of the method expressed as relative standard deviation (RSD) was near ±2%, indicating the accuracy of this method.

X.7. Determination of the Antioxidant Activity of Verbascoside by DPPH Method

We analyzed the verbascoside's ability to neutralize free radicals by using the DPPH test, the percent reduction capacity of DPPH radicals being calculated according to the equation presented in previous studies. The results obtained are reported as the average of the percentage inhibition values for all sample solutions analyzed and are presented in Table X.3.

Table X.3. *Determination of antioxidant activity of the studied EVOO samples*

EVOO Samples	% Inhibition - DPPH
Pietro Coricelli	10,31
TopSeller Oil	13,02
Regina	14,44
Mazza	18,67
Olitalia	21,17
Costa d'Oro Il Grezzo	16,28
Minerva	22,91
Costa D'Oro L'extra	24,64
Monastiri	20,30
Rivano Olio	20,62

Table X.3 shows that the highest percentage inhibition value was obtained for Costa D'Oro L'extra (Italy), this result being in agreement with the value obtained in the quantitative determination of verbascoside in EVOO samples, where the highest amount of verbascoside contained per kilogram EVOO was also achieved for this matrix

X.8. Conclusions

This study reported, for the first time, the fabrication of an electrochemical sensor by modifying a GO-based SPCE with a pentapeptide containing the NH_2-FESNF-CO-NH_2 sequence, used for the accurate determination of verbascoside from real matrices with complex composition, i.e., samples of different EVOOs. Applying CV as a detection technique has demonstrated very good analytical performance with applicability in laboratory practice. Kinetic studies of electrochemical processes confirmed the superiority of the modified SPCE/GO-Pentapeptide sensor over the unmodified SPCE/GO sensor, obtaining with SPCE/GO-Pentapeptide a wide range of linearity (0.1–10.55 µM) and a low detection limit (9.38×10^{-8} M), respectively. In addition, the influence of other related compounds on the voltammetric response was reduced due to the favorable specificity of the newly developed sensor. Moreover, the antioxidant activity of the compound of interest was achieved by DPPH spectrophotometric assay, thus asserting the antioxidant activity of verbascoside in EVOO samples.

CHAPTER XI. GENERAL CONCLUSIONS

Studies conducted in recent years on the importance of consuming antioxidant-rich foods for the proper functioning of the body and to maintain a healthy body, both indoors and outdoors, have concluded that the current lifestyle, stress, sedentary, pollutants, and the environment are not only a part of the body, but also a part of the body. as well as other factors change the need for these substances, which leads to the need to supplement the diet with the products that supplement their intake, namely nutritional supplements and to use cosmetics that provide protection against free radicals. The nutraceutical formulations available on the pharmaceutical market can be found in different forms of presentation, their complex composition in biologically active compounds being, most of the times, presented only from a qualitative point of view, without any details on the quantity or concentration of those compounds. Also of particular importance is the proportion of active principles in cosmetic formulations, the latter providing a wide range of health and beauty benefits for the skin.

Therefore, the main objective of this paper was to develop and optimize the applicability of new methodologies for determining active compounds and evaluating the antioxidant activity of some nutraceuticals and cosmetics, using chemically modified sensors and biosensors in the laboratory with different nanomaterials, peptides and enzymes. Comparative analyzes of the values obtained by applying electrochemical methods were also performed and those resulting from the application of spectrophotometric methods for determining antioxidant activity, such as DPPH, ABTS or galvinoxil.

The thesis includes a current theoretical study, drawn up on the basis of detailed bibliographic documentation through the specialized literature, respectively the analysis of achievements in our field of interest, mentioned in the second part of the paper.

The information obtained through the studies discussed in this first part of the thesis showed that:

- Analytical methods for determining antioxidant activity are based on chemical reactions, and the evaluation of kinetics or steady state is carried out using spectrophotometry.
- As complementary methods for such spectrophotometric studies, methods based on sensors and electrochemical biosensors can be used, calibration and validation steps are required. The development of sensors and biosensors has created a wide variety of new possibilities for detection, as well as opportunities for efficient use of electroanalysis, with remarkable analytical characteristics, which can become useful tools in clinical diagnosis through their simple, selective, rapid and sensitive determinations.
- The use of chemical methods, along with electrochemical methods, can lead to elucidation of the mechanisms of action and the kinetics of the processes in which antioxidants are involved.
- Phenolic compounds are considered true antioxidants, being associated with benefits in reducing the risks of various degenerative diseases associated with oxidative stress by protecting cellular constituents against oxidative damage.

The second part of this research paper, respectively the practical part, is represented by the personal contributions materialized in experimental studies carried out on the basis of everything that was studied and analyzed during the scientific documentation stage. Thus, electrochemical analyzes and determinations were performed on the antioxidant activity of

selected nutraceutical, cosmetic and food products, having in composition the bioactive compounds of interest, namely chlorogenic acid, catechin, rosmarinic acid and verbascoside. In these experimental studies, screen-printed sensors based on chemically modified carbon nanomaterials were used in the laboratory, which proved excellent properties, both used as such and after modification with other chemical mediators, enzymes or peptides. This research brought originality to the thesis and led to the following conclusions:

- The studies carried out to characterize and determine the bioactive compounds were carried out by means of voltammetric methods of analysis, namely CV, SWV and DPV. They contributed to the achievement of remarkable original results by optimizing working parameters for a stable signal, namely the scan speed, the potential range, the pH of the solutions, the amount of modifier material, as well as its concentration.

- In order to observe changes in the sensors obtained in the laboratory based on chemical mediators and peptides, two methods were used, namely SEM and FT-IR spectrometric method. In the case of biosensors obtained by immobilizing enzymes on the surface of the voltametric sensors, the EIS method was also used in addition to the two techniques. It evaluated the limiting factors in relation to load transfer and diffusion in the system for each biosensor.

- Specific enzymes can be used effectively to selectively identify target compounds important in food and pharmaceutical quality control. Lacase and tyrosinase are the two enzymes most commonly used to monitor antioxidant activity, especially phenolic compounds, and are able to oxidize them to quinonic compounds, which are electroactive.

- Peptides have been used as sensitive components for the manufacture of new sensors capable of assessing and quantifying the content of antioxidant compounds in food samples represented by olive oils or cosmetics, with minimal sample preparation, using as a support SPCEs, Modify with a composite go film.

- The electrochemical methods were used to determine the antioxidant activity of the samples to be analyzed, more precisely based on the main electrochemical parameters (EPA and IPA) obtained from voltammetric measurements, with the achievement of very good correlations between these data and the results by applying spectrophotometric methods for the analysis of the respective samples.

The changes made to electrochemical devices in the laboratory have been very successful in terms of the increased performance obtained later on in voltammetric determinations, proving their effectiveness for quality control of nutraceutical formulations, food products, their applicability may be extended in the future to other types of real evidence, such as biological or environmental samples.

CHAPTER XII. FUTURE RESEARCH PERSPECTIVES

Based on the research carried out with these versatile devices, namely chemically modified sensors and enzyme biosensors developed in the laboratory, new directions in their development for determining antioxidant activity may be related to the use of new nanomaterials or polymer compounds as mediators, the use of more stable and smaller immobilization platforms or multienzymatic systems, the application of chemometric devices in the evaluation of experimental data and the development of disposable portable biosensors. In addition, improving the electrochemical characteristics of newly manufactured devices through additional intermediate steps can lead to increased capability of operation as sensors or biosensors in various environments.

- In relation to the results obtained with sensors and biosensors for the detection of chlorogenic acid, catechin, rosmarinic acid and verbascoside, results that are comparable and, in some cases, better than those reported in the literature of specialists, this elaborate doctoral paper opens new perspectives on:

- Expanding the range of phenolic compounds analyzed, from different samples and in different combinations, so that the research focuses mainly on their simultaneous detection.

- Obtaining new sensors and biosensors by modification with other nano-composites (polymers, optical fibers) or biological materials (aptamers, DNA or proteins) and widening the analysis spectrum by other methods, such as linear scan voltammetry or chronoamperometry.

- Characterization of the electrode surfaces in order to obtain information about the changes made to them by methods such as TEM, AFM, etc.

The application of multi-dimensional data analysis techniques that can be extremely useful for studying the similarities and differences between foods, on the one hand, and antioxidants, on the other hand, as well as establishing the compounds characteristic of each food class.

Therefore, there is significant potential in this field of research in order to develop new sensitive devices, connected to smart electronic platforms for the detection of different analytes in the body or for the routine testing for the quality control of pharmaceutical products, thus providing important perspectives for numerous biomedical, pharmaceutical or industrial applications.

CHAPTER XIII. VALORIZATION AND IMPACT OF RESEARCH RESULTS

XIII.1. Published articles in ISI listed journals

XIII.1.1. Published articles from the doctoral thesis

- **2021**

1. Munteanu, I.G.; Apetrei, C. Analytical Methods Used in Determining Antioxidant Activity: A Review. *Int. J. Mol. Sci.* **2021**, *22*, 3380, https://doi.org/10.3390/ijms22073380, Impact factor 4,556, O1;

2. Munteanu, I.-G.; Apetrei, C. Electrochemical Determination of Chlorogenic Acid in Nutraceuticals Using Voltammetric Sensors Based on Screen-Printed Carbon Electrode Modified with Graphene and Gold Nanoparticles. *Int. J. Mol. Sci.* **2021**, *22*, 8897. https://doi.org/10.3390/ijms22168897, Impact factor 5,924, Q1;

3. Munteanu, I.G.; Apetrei, C. A Review on Electrochemical Sensors and Biosensors Used in Chlorogenic Acid Electroanalysis. *Int. J. Mol. Sci.* **2021**, *22*, 13138. https://doi.org/10.3390/ijms222313138, Impact factor 5,924, Q1;

- **2022**

4. Munteanu, I.G.; Apetrei, C. A Review on Electrochemical Sensors and Biosensors Used in Assessing Antioxidant Activity. *Antioxidants* **2022**, *11*, 584. https://doi.org/10.3390/antiox11030584, Impact factor 6,313, Q1;

5. Munteanu, I.G.; Apetrei, C. Tyrosinase-Based Biosensor—A New Tool for Chlorogenic Acid Detection in Nutraceutical Formulations. *Materials* **2022**, *15*, 3221. https://doi.org/10.3390/ma15093221, Impact factor 3,623, Q1;

6. Munteanu, I.G.; Apetrei, C. Assessment of the Antioxidant Activity of Catechin in Nutraceuticals: Comparison between a Newly Developed Electrochemical Method and Spectrophotometric Methods. *Int. J. Mol. Sci.* **2022**, *23*, 8110. https://doi.org/10.3390/ijms23158110, Impact factor 6,208, Q1;

7. Munteanu, I.G.; Grădinaru, V.R.; Apetrei, C. Sensitive Detection of Rosmarinic Acid Using Peptide-Modified Graphene Oxide Screen-Printed Carbon Electrode. *Nanomaterials* **2022**, *12*, 3292. https://doi.org/10.3390/nano12193292, Impact factor 5,719, Q1;

8. Munteanu, I.G.; Grădinaru, V.R.; Apetrei, C. Development of a Chemically Modified Sensor Based on a Pentapeptide and Its Application for Sensitive Detection of Verbascoside in Extra Virgin Olive Oil. *Int. J. Mol. Sci.* **2022**, *23*, 15704. https://doi.org/10.3390/ijms232415704, Impact factor 6,208, Q1.

XIII.1.2. Published articles related to the research topic

- **2023**

9. Munteanu, I.G.; Apetrei, C. Classification and Antioxidant Activity Evaluation of Edible Oils by Using Nanomaterial-Based Electrochemical Sensors. *Int. J. Mol. Sci.* **2023**, *24*, 3010. https://doi.org/10.3390/ijms24033010, Impact factor 6,208, Q1.

Cumulative impact factor: 50,683 WOS

XIII.2. Papers and posters presented at international and national conferences

- **2020**

1. Irina – Georgiana Bulgaru (Munteanu), Constantin Apetrei, Detection of p-coumaric acid with electrochemical sensors, International online Conference – 8th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 259, Galați, Romania, June 18-19, 2020, **poster;**

2. Irina – Georgiana Bulgaru (Munteanu), Dorin Dăscălescu**,** Constantin Apetrei, Nanocomposite sensor for sensitive detection of catechol, International online Conference – 8th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 259, Galați, România, June 18-19, 2020, **poster;**

3. Ancuța Dinu, Dorin Dăscălescu, **Irina – Georgiana Munteanu**, Alexandra Virginia Bounegru, Ramona-Oana Roșca, Constantin Apetrei, Electrochemical sensors based on nanomaterials employed in water analysis, 8th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 258, Galați, Romania, June 18-19, 2020, **poster**;

4. Irina – Georgiana Bulgaru (Munteanu), Constantin Apetrei, Electrochemical determination of catechol based on carbon electrode modified with graphene and gold nanoparticles, The 5th International Conference „New Trends on Sensing-Monitoring-Telediagnosis for Life Science NT-SMT-LS 2020", Book of abstracts, pp.106, Brașov, România, July 3-4, 2020, **poster**.

- **2021**

5. Irina – Georgiana Bulgaru (Munteanu), Constantin Apetrei, Detection of chlorogenic acid with electrochemical sensors, International online Conference – 9th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 270, Galați, România, June 10-11, 2021, **poster;**

6. Irina – Georgiana Munteanu, Constantin Apetrei, Electrochemical determination of chlorogenic acid in pharmaceutical products, The 1st International Electronic Conference on Chemical Sensors and Analytical Chemistry, July 1-15 2021, **poster**;

7. Constantin Apetrei, Alexandra Virginia Bounegru, **Irina - Georgiana Munteanu**, Irina Mirela Apetrei, Electrochemical sensors and biosensors based on polypyrole for detection of phenolic compounds in olive oils, International online Conference – 9th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 117, Galați, România, June 10-11, 2021, **oral presentation;**

8. 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, The 1st International Electronic Conference on Chemical Sensors and Analytical Chemistry, July 1-15, 2021, **poster**;

9. Ancuța Dinu, Constantin Apetrei, Dorin Dăscălescu, **Irina-Georgiana Munteanu (Bulgaru)**, Ramona-Oana Roșca (Gunache), Detection of Amino Acids L-Phenylalanine, L-Tyrosine and L-Tryptophan with Biosensors based on Polypyrrole, Exploratory Workshop NeXT-Chem III, Book of abstracts, pp. 11, May 27-28, 2021, București, Romania, **oral presentation**;

10. Alexandra Virginia Bounegru (Mereșescu), Constantin Apetrei, **Irina – Georgiana Munteanu (Bulgaru)**, Ramona – Oana Roșca (Gunache), Development of biosensors for the hydroxycinnamic acids analysis, Exploratory Workshop NeXT-Chem III, Book of abstracts, pp. 10, May 27-28, 2021, București, Romania, **oral presentation**;

- **2022**

11. Irina – Georgiana Bulgaru (Munteanu), Constantin Apetrei, Sensitive properties of a screen printed carbon electrode modified with graphene, manganese phthalocyanine and tyrosinase for voltammetric detection of chlorogenic acid, 10th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 92, Galați, Romania, June 9-10, 2022, **oral presentation;**

12. Irina – Georgiana Bulgaru (Munteanu), Constantin Apetrei, Laccase biosensors based on screen-printed electrode modified with carbon nanotubes and gold nanoparticles for catechin detection in nutraceutical formulations, 10th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 243, Galați, Romania, June 9-10, 2022, **poster;**

13. Alexandra Virginia Bounegru, **Irina - Georgiana Munteanu**, Constantin Apetrei, Development of an electroanalytical method for detecting adulteration of extra virgin olive oils, 10th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 41, Galați, Romania, June 9-10, 2022, **oral presentation;**

14. Irina – Georgiana Bulgaru (Munteanu), Constantin Apetrei, Comparative study on the antioxidant activity of extra virgin olive oil samples using a newly developed electrochemical method and DPPH spectrophotometric assay, The 6th International Conference „New Trends on Sensing-Monitoring-Telediagnosis for Life Science NT-SMT-LS 2022", Book of abstracts, Brașov, Romania, September, 8-10, 2022, **poster**;

15. Irina – Georgiana Munteanu, Constantin Apetrei, Electroanalytical method for determination of rosmarinic acid based on chemically modified sensors, The International Symposium PRIOrities of CHEMistry for a sustainable development, Priochem, Book of Abstracts, pp. 24, București, Romania, October, 26-28, 2022, **oral presentation**;

16. Constantin Apetrei, Andreea Loredana Comănescu, Andrei Daniel Geman, **Irina Georgiana Munteanu**, Alexandra Virginia Bounegru, Irina Mirela Apetrei, Elisabeta Irina Geană, Electrochemical (bio)sensor array coupled with multivariate data analysis for the assessment of virgin olive oil quality, The International Symposium PRIOrities of CHEMistry for a sustainable development, Priochem, Book of Abstracts, pp. 11, București, Romania, October, 26-28, 2022, **oral presentation**;

- **2023**

17. Irina Mirela Apetrei, Alexandra Virginia Bounegru, **Irina Georgiana Munteanu**, Constantin Apetrei, Detection of olive oils adulteration with electrochemical sensors and biosensors based on nanomaterials and enzymes, The 18th International Conference of Constructive Design and Technological Optimization in Machine Building Field, OPROTEH 2023, Book of Abstracts, pp. 79, Bacău, România, May, 11-13, 2023, **prezentare orală**.

18. Irina – Georgiana Munteanu, Constantin Apetrei, Electrochemical peptide-based Sensor for direct detection and quantification of verbascoside in extra virgin olive oil, 11th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos"

University of Galați, Book of abstracts, pp. 106, Galați, România, June 8-9, 2023, **prezentare orală;**

19. Irina – Georgiana Munteanu, Constantin Apetrei, Nanomaterial-based electrochemical sensors for antioxidant activity evaluation and discrimination of vegetable oils, 11th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 106, Galați, România, June 8-9, 2023, **prezentare orală.**

XIII.3. Awarding research results

- **2020**

Third Prize - Irina – Georgiana Bulgaru (Munteanu), Dorin Dăscălescu**,** Constantin Apetrei, Nanocomposite sensor for sensitive detection of catechol, International online Conference – 8th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 259, Galați, România, June 18-19, 2020, **poster.**

- **2021**

Second Prize - GALA CEREX IOSUD UDJG (Gala Cercetării de Excelență – Premierea rezultatelor cercetării științifice doctorale – Universitatea „Dunărea de Jos" din Galați) - Munteanu, I.G.; Apetrei, C. Analytical Methods Used in Determining Antioxidant Activity: A Review. *Int. J. Mol. Sci.* **2021**, *22*, 3380, https://doi.org/10.3390/ijms22073380, Impact factor 4,556, Review;

First Mention - Ancuța Dinu, Constantin Apetrei, Dorin Dăscălescu, **Irina-Georgiana Munteanu (Bulgaru)**, Ramona-Oana Roșca (Gunache), Detection of Amino Acids L-Phenylalanine, L-Tyrosine and L-Tryptophan with Biosensors based on Polypyrrole, Exploratory Workshop NeXT-Chem III, Book of abstracts, pp. 11, May 27-28, 2021, București, România, **Online International Conference**, **oral presentation.**

- **2022**

First Prize - Irina – Georgiana Bulgaru (Munteanu), Constantin Apetrei, Sensitive properties of a screen printed carbon electrode modified with graphene, manganese phthalocyanine and tyrosinase for voltammetric detection of chlorogenic acid, 10th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 92, Galați, România, June 9-10, 2022, **oral presentation;**

PRIOCHEM 2022 AWARD for the work entitled: "Electroanalytical Method for Determination of Rosmarinic Acid Based on Chemically Modified Sensors", **Irina – Georgiana Munteanu,** Constantin Apetrei**, oral presentation;**

First Prize - GALA CEREX IOSUD UDJG (Gala Cercetării de Excelență – Premierea rezultatelor cercetării științifice doctorale – Universitatea „Dunărea de Jos" din Galați)- Munteanu, I.G.; Apetrei, C. A Review on Electrochemical Sensors and Biosensors Used in Assessing Antioxidant Activity. *Antioxidants* **2022**, *11*, 584. https://doi.org/10.3390/antiox11030584, Impact factor 6,313, Review.

- **2023**

First Prize - Irina – Georgiana Munteanu, Constantin Apetrei, Nanomaterial-based electrochemical sensors for antioxidant activity evaluation and discrimination of vegetable oils, 11th Edition of Scientific Conference organized by the Doctoral Schools of „Dunărea de Jos" University of Galați, Book of abstracts, pp. 106, Galați, România, June 8-9, 2023, **oral presentation;**

XIII.4. Related activities carried out within the individual program of doctoral university studies

✓ **Research assistant within the project** "New biosensors and intelligent tools for the ultrasonic detection of olive oil falsification"**, PN-III-P4-ID-PCE-2020-0923,** period 10.03.2021-31.12.2023.

✓ **Member of the target group** within the project "Program for increasing performance and Innovation in Excellence Doctoral and Postdoctoral Research" – PROINVENT, co-financed by the European Social Fund through the Human Capital Operational Program, 2014-2020, contract no. 62487/03.06.2022, POCU/993/6/13 – SMIS Code: 153299**.**

✓ **Member of the European Center of Excellence for the Environment (ECEE)** team.

✓ Participation in courses, seminars, workshops, internships organized by the Doctoral School of Fundamental Sciences and Engineering, as well as those within the PROINVENT project.

SELECTIVE BIBLIOGRAPGHY

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