Estimation of Active Compounds Quantity from Pharmaceuticals Based on *Ginkgo biloba*

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Received: 29 September 2020; Accepted: 3 November 2020; Published: 4 November 2020

**Abstract:** *Ginkgo biloba* is one of the most important sources of active compounds, mainly flavonoids and phenolic compounds. Due to its importance related to pharmaceutical practice, the making of a qualitative and quantitative method for the detection and quantification of active compounds from *Ginkgo biloba* pharmaceutical products is desirable. In this study, the content of biological active compounds from *Ginkgo biloba* products was estimated using cyclic voltammetry. The electrochemical determination of active compounds was carried out by using a screen-printed carbon electrode modified with carbon nanotubes. The studies regarding parameter optimization were made using solutions containing potassium ferrocyanide and catechol, respectively. In both cases, the redox processes of studied compounds was observed, which were controlled by the diffusion phenomenon. We analyzed two pharmaceutical products containing *Ginkgo biloba*, a RX product (recipe medicine requires a medical prescription to be dispensed) and an OTC (Over-The-Counter, which can be obtained without a prescription) product. The cyclic voltammograms of the two products showed two redox processes due to the antioxidant properties of the products. It was found that the RX product had a greater content of active compounds compared to the OTC product. Therefore, the voltammetric method has great utility for the determination of compounds with redox properties from pharmaceutical products containing *Ginkgo biloba*.

**Keywords:** Cyclic Voltammetry; carbon nanotubes; electrochemical sensor; *Ginkgo biloba*

1. Introduction

*Ginkgo biloba* is one of the main sources of active substances such as flavonoids and phenolic compounds [1]. It is considered to be a frequently used plant, specifically as an expectorant, anti-asthmatic, anti-inflammatory, sedative, and antithrombotic [2]. According to the European Drug Agency, *Ginkgo biloba* administration is indicated for treating several symptoms like failure to maintain attention, memory loss, dementia, chronic cerebrovascular insufficiency, peripheral arterial disease, etc. [3]. Due to its importance in pharmaceutical practice, a qualitative and quantitative method has been searched for the detection and quantification of active compounds from *Ginkgo biloba* based products [4]. In pursuing this goal, numerical analytical methods have been developed for the determination of the antioxidant capacity such as chemical methods, spectrophotometric (Folin–Ciocalteu method, determination of antiradicalic capacity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) or 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)), and electrochemical methods based on cyclic voltammetry [5,6]. As a result, the pharmaceutical products based on *Ginkgo biloba* that are produced with antioxidant properties can be analyzed with electrochemical methods [7]. These methods can be used in routine analysis because they are fast, easy to carry out, and inexpensive [8]. The electrochemical sensors based on screen-printed electrodes have great importance in the electroanalysis field due to their advantages compared to classical electrodes. The most important
ones are the miniaturization of the electrochemical cell, the reproducibility of the fabrication process, and the feasibility of being used in screening analysis [9]. Voltammetric methods have been developed for the determination of the antioxidant capacity of *Ginkgo biloba* using carbon-based electrodes [10]. Nevertheless, the electrochemical performance of carbon electrodes can be enhanced by modifying their surfaces using carbonaceous nanomaterials such as carbon nanotubes [11,12], carbon nanofibers [13], graphene [14], fullerene [15], biochar [16,17], etc. These approaches have been used extensively for the modification of screen-printed electrodes and development of electrochemical sensors [18]. The carbon nanotubes are considered to be carbon allotropes with a cylindrical nanostructure and are utilized in many domains due to enhanced thermal conductivity and improved electro-mechanical properties [11]. Carbon nanotubes offer characteristics such as high electrical conductivity, high surface area, and good chemical stability [19]. Additionally, the carbon nanotubes (CNTs) enhance the rate of electron transfer when used as an electrode-modifying material [18]. These important features are important in the sensing and study of antioxidant properties of natural substances is still challenging.

The objective of this study was to estimate the antioxidant capacity of pharmaceutical products based on *Ginkgo biloba* using a sensor based on a screen-printed carbon electrode modified with carbon nanotubes using cyclic voltammetry as the detection technique. The correlation with antioxidant activity determined by the DPPH method will be used for the validation of the electrochemical method.

2. Materials and Methods

2.1. Chemicals and Solutions

All compounds were of highest purity available; therefore these were utilized without additional purification. The studies regarding the optimization of experimental parameters were conducted using standard potassium ferrocyanide and catechol, respectively, obtained from Sigma-Aldrich (St. Louis, MO, USA). The electrolytes of support used for the electrochemical experiment were 0.1 M potassium chloride, potassium ferrocyanide 0.001 M, and catechol 0.001 M. All solutions were prepared with ultra-pure water, MilliQ water (resistivity 18.2 MΩ·cm) obtained from a water purification system (Milli-Q Simplicity®, Bedford, MA, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ethanol (96%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Electrochemical Measurements

The screen-printed carbon electrodes (the diameter of the electrode is 4 mm) were purchased from Dropsens Ltd. (Llanera, Spain). The carbon nanotubes (CNTs), single-walled (≥95% as carbon nanotubes) with a 0.78 nm average diameter that were used for the modification of screen-printed carbon electrodes (SPCE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the modification of the screen-printed carbon electrodes, the following procedure was carried out. The dispersion of CNTs was prepared by mixing 1 mg CNT with 1 mL methanol followed by ultrasonication for 2 h. Through this method, a homogeneous dispersion of CNTs was obtained. The dispersion was deposited on the working electrode by the casting method followed by the evaporation of the solvent at room temperature in a desiccant [20]. The slow evaporation of the solvent is important in obtaining the self-assembly of the CNT in an ordered manner. The characterization of the sensor modified with CNTs was carried out by scanning electron microscopy (scanning electron microscope FlexSEM 1000, Hitachi, Tokyo, Japan). A high degree of order of CNTs can be observed in the SEM image (Figure 1f).
All of the electrochemical measurements (by cyclic voltammetry) were carried out using a potentiostat/galvanostat EG&G (Princeton Applied Research, Oak Ridge, TN, USA) controlled by a Windows operating computer with the ECHEM software. It was utilized for the characterization of the electrode signals and to electroanalyze the products containing Ginkgo biloba. A three electrode system was put to use. The working electrode was the SPCE modified with CNTs (SPCE-CNT). The reference electrode was the silver electrode/silver chloride (KCl 3 M) and the counter-electrode was represented by a platinum wire. Thus, the connections to the potentiostat/galvanostat were made using independent cables for each electrode. All potentials indicated in this study were reported to the reference electrode Ag/AgCl (KCl 3 M). The cyclic voltammograms were registered in a potential ranging from −0.4 to +0.7 V. In general, the scan rate can usually range from 0.1 to 1.0 V·s⁻¹. In this study, in order to stabilize the electrochemical signal with the help of cyclic voltammetry, three successive cycles with scan rate of 0.1 V·s⁻¹ were registered. Furthermore, two successive cycles were registered with the same scan rate. In order to study the influence of scan rate on the signal characteristics, we used values ranging from 0.1 to 1.0 V·s⁻¹.

2.3. Pharmaceutical Analysis

The sensor applicability was studied by analyzing some commercial products based on Ginkgo biloba. These analyzed, in the optimal experimental condition, two pharmaceutical products containing Ginkgo biloba, a RX product and an OTC product, which were purchased from local drug stores. One was Tanakan® (Munich, Germany), which contains 40 mg dry refined and quantified extract of Ginkgo biloba and the other one was GinkoPrim Max® (Bucharest, Romania), which contains 120 mg of standardized Ginkgo biloba extract, 150 mg Mg, and 15 mg Zn. One tablet was triturated and then dissolved in 100 mL KCl 0.1 M. The solution was homogenized and dissolved using the Elma S10H Elmasonic equipment (Elma Schmidbauer GmbH, Singen, Germany). Furthermore, the solution was

Figure 1. (a) Image of screen-printed carbon electrode; (b) Image of potentiostat/galvanostat EG&G (Princeton Applied Research); (c) Image of ECHEM software; (d) Image of electrochemical cell containing GinkoPrim Max solution; (e) Schematic representation of the screen-printed carbon electrode modified with carbon nanotubes; (f) SEM image of the sensitive element of the CNT modified sensor.
introduced into the electrochemical cell and connected the three electrodes (the working electrode, the reference electrode and the counter electrode). All of the pharmaceutical samples were analyzed in triplicate. All of the procedures and experimental protocols were used according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Figure 1 shows images of the screen-printed working electrode (a), the potentiostat/galvanostat EG&G (b), the Echem software interface for the experimental controlling and data acquisition (c), the electrochemical cell for the analysis of the GinkoPrim Max sample (d), and the schematic representation of the SPCE (screen-printed carbon electrode) modified with CNTs (e).

2.4. Antioxidant Activity (DPPH Free Radical Scavenging Activity)

The DPPH assay is a fast, simple, and commonly used method to evaluate the antioxidant properties of compounds or vegetable extracts, and is known to be sensitive toward different classes of antioxidant compounds such as phenolic compounds [21]. The scavenging capacity of antioxidants toward DPPH free radicals is quantified by the decrease in absorbance at 517 nm [22]. This assay provides information on the antioxidants’ capacity to donate electrons or hydrogen atoms scavenging the DPPH free radical [23].

The DPPH stock solution of 0.1 mM was prepared from the DPPH reagent and 96% ethanol and kept at room temperature in the dark. A total of 0.5 mL of each sample solution (the same solutions used in the electrochemical measurements) was added to 2 mL of DPPH solution separately. These solution mixtures were kept in the dark for 30 min at room temperature, then the absorbance was measured.

Experimental data were acquired on a Rayleigh UV2601 UV/Vis double-beam spectrophotometer (Beijing Beifen-Ruili Analytical Instrument, Beijing, China) set at 517 nm by measuring the sample absorbance decrease against the control (blank solution). The DPPH radical scavenging capacity result in discoloration is calculated as a percentage reduction; lower absorbance of the reaction mixture indicated higher free radical scavenging activity of DPPH according to the following equation [21]:

\[
\text{% DPPH Reduction} = 100 \times \left( \frac{-AE}{AD} \right)
\]

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

The % DPPH reduction values were normalized per tablet for both pharmaceutical samples. All assays were carried out in triplicate and the results are reported as the average. The relative standard deviation of the results was close to 1%.

3. Results and Discussion

The cyclic voltammetry measurements can be used for the determination of antioxidant properties of a large range of compounds [22]. These properties depend on the chemical structure of the compounds and their redox characteristics [23]. Therefore, in this study, a series of pharmaceutical products based on Ginkgo biloba were analyzed by cyclic voltammetry and the antioxidant properties were correlated with the antioxidant capacity determined by the DPPH assay.

The preliminary studies were carried out using standard compounds with redox properties such as potassium ferrocyanide and catechol, respectively. The voltammetric behavior of the modified screen-printed electrodes with carbon nanotubes (SPCE-CNT) was studied in order to evaluate the electroactive surface of the working electrode. Initially, the cyclic voltammogram of the modified screen-printed electrode with carbon nanotubes was registered using a \( K_4[Fe(CN)_6] \) solution of 0.001 M (Figure 2).

In Figure 2, an anodic peak and a cathodic peak can be observed, which were related to the reversible redox processes of the ferrocyanide ion on the surface of the working electrode, as shown by Equation (2):

\[
[Fe(CN)_6]^{4-} \leftrightarrow [Fe(CN)_6]^{3-} + e^-
\]
The oxidation peak (anodic) occurred at \( E_{pa} = 265.65 \text{ mV} \), and the reduction peak (cathodic) occurred at \( E_{pc} = 81.15 \text{ mV} \). The currents that corresponded to the two peaks were \( I_{pa} = 22.12 \mu\text{A} \) and \( I_{pc} = 18.41 \mu\text{A} \). The formal redox potential \( (E^0) \) of the reversible couple was equal to: \( E^0 = (E_{pa} + E_{pc})/2 \), thus 173.4 mV in this case; this value is in agreement with other results published in the literature [9]. According to \( \Delta E = E_{pa} - E_{pc} = 184.5 \text{ mV} \) and \( I_{pc}/I_{pa} = 0.83 \), it can be stated that the electrochemical process of the ferrocyanide ion at the surface of the working electrode is quasi-reversible [9]. As can be observed in Figure 2, the unmodified SPCE showed redox peaks corresponding to the ferrocyanide-ferricyanide couple. However, the currents were lower and the peak potentials shifted greater (anodic peak) and lower (cathodic peak) when compared with the peaks observed in the case of SPCE-CNT. These results prove the increment of the sensitivity performances of the SPCE when it is modified with CNTs.

\[
\begin{align*}
\Delta I &= \frac{I_{pa} - I_{pc}}{2} \\
\Delta E &= \frac{E_{pa} + E_{pc}}{2} \\
E^0 &= \frac{E_{pa} + E_{pc}}{2}
\end{align*}
\]

The % DPPH reduction values were normalized per tablet for both pharmaceutical products based on Ginkgo biloba. These properties depend on the chemical structure of the ferrocyanide ion at the surface of the working electrode is quasi-reversible [9]. As can be observed in Figure 3.

Following these results, it is safe to say that the screen-printed carbon electrode modified with carbon nanotubes is useful in the electroanalysis of active redox compounds, organic or inorganic, with very good sensibility.

\[\text{(3)}\]

\[
\begin{align*}
\text{catechol} &\rightarrow \text{quinine} \\
\text{2H}^+ + 2e^- &\rightarrow \text{H}_2
\end{align*}
\]
In order to obtain the determining factor of the kinetics of the electrochemical processes, the influence of scan rate on the voltammetric response of the sensor immersed in the potassium ferrocyanide solution was studied. This study was carried out by registering the cyclic voltammograms in the range 0.1–1.0 V·s\(^{-1}\). The results are presented in Figure 4.

![Figure 3. Cyclic voltammograms of the SPCE-CNT (solid line) and SPCE (dashed line) immersed in a 0.001 M catechol solution registered with a scan rate of 0.1 V·s\(^{-1}\).](image)

![Figure 4. (a) Cyclic voltammograms of SPCE-CNT immersed in a 0.001 M K\(_4\)[Fe(CN)]\(_6\) solution registered with scan rates in the range 0.1–1.0 V·s\(^{-1}\). (b) The relationship between the anodic peak current values and the square root of the scan rates.](image)
In Figure 4a, the increase of the current peaks concomitant with the increase of the scan rate can be observed. For the electrochemical reactions, the current peak can be described by the Randles–Sevcik equation:

\[
I_p = 0.4463nFAc(nF \varepsilon D/RT)^{1/2}
\]

where \( n \) represents the number of electrons involved in the redox exchange; \( c \) is the analyte concentration (M); \( v \) is the scan rate (V s\(^{-1}\)); \( F \) is the Faraday constant (96,485 C mol\(^{-1}\)); \( A \) represents the electrode surface in cm\(^2\); \( R \) is the universal constant of gas (8.314 J mol\(^{-1}\) K\(^{-1}\)); \( T \) is the absolute temperature (K); and \( D \) is the diffusion coefficient (cm\(^2\) s\(^{-1}\)) [24,25].

According to the Randles–Sevcik equation, the peak current is directly proportional with the analyte concentration and also depends upon the scan rate.

By representing the anodic peak currents versus the square root from the scan rates, a linear dependence was obtained with a determination coefficient \( R^2 \) equal to 0.9997. From this statement, combined with the Randles–Sevcik equation [26], it can be concluded that the process of electrochemical oxidation is controlled by the diffusion process of the potassium ferrocyanide from the solution at the surface of the working electrode.

In order to register the voltammetric responses of the sensor in the solutions of the pharmaceutical products, cyclic voltammetry with potential ranging from −0.4 V to +0.7 V was used. Figure 5 presents the cyclic voltammograms of SPCE-CNT immersed in the Tanakan solution and GinkoPrim Max solution, respectively, registered with a scan rate of 0.1 V s\(^{-1}\).

![Figure 5. Cyclic voltammograms of SPCE-CNT immersed in Tanakan solution (purple line) and GinkoPrim Max (blue line).](image)

In the cases of both pharmaceutical products, two peak pairs could be observed, which was better highlighted and more intense in the case of the pharmaceutical product Tanakan. These peaks were related to the presence of compounds with antioxidant properties from the pharmaceutical products mentioned below. In accordance to the data from the literature, the main classes of compounds with antioxidant properties from Ginkgo biloba were the terpenoids and flavonoids [27].

The main compounds from the phytochemical constituents of the Ginkgo biloba leaves are terpenoids and flavonoids. The primary terpenoids represented by the ginkgolides A, B, C, and bilobalides as well as the flavonoids quercetin, kaempferol, and isorhamnetin, all have biological and pharmacological properties. The ginkgolides have the same molecular geometry, but they differ from each other, having a distinct number and site of the hydroxyl groups, while the flavonoids are often glycoside derivatives. Quercetin is metabolized, leading to kaempferol, while isorhamnetin is the metabolic result of kaempferol. Most of the Ginkgo biloba extracts available on the market are made from water–acetone or ethanol–water, enriched with leaves [28]. Most parts of Ginkgo biloba extracts are flavonoid derivatives or terpene triacetates. The commercial extracts contain 22–27% flavone-glycosides and 57% lactone-terpenes, of which 2.8–3.4% are ginkgolides A, B, C; 2.6–3.2% are bilobalides; and ginkgolic
acids in a very small amount [29]. It seems that the alkylphenol and the alkylbenzoic acid derivative are eliminated from the extract due to their allergenic and immunotoxic properties. The terpenes-trilactones are rare in the vegetal kingdom and until this point, they have been identified only in Ginkgo biloba. The structure of these active compounds with antioxidant properties are presented in Figures 6–8.

![Figure 6. Chemical structure of Ginkgolide.](image)

![Figure 7. Chemical structure of Bilobalide.](image)

The pharmacological effect of ginkgolides B varies, although the antagonistic activity of the thrombocyte activation factor represents their main role. This effect is an important factor in many research subjects in biology and pharmacology, which concluded that the RGb-761 extract is one of the most used drug in phytomedicine. EGb-761 represents a 24% standardized flavonoid glycoside blend, 6% terpenes-trilactones, the bilobalide as the active ingredient of Tanakan, and also of many other pharmaceutical extracts. The pharmacological effect of Tanakan is in accordance with the neuroprotective and vasodilatory properties. The neuroprotective effect is due to the antioxidant and free radical activity, which can contribute to a moderate anxiolytic and antidepressant effect [30–33]. Bilobalide (Figure 7) is a sesquiterpenoid lactone contained in the Ginkgo biloba extract. Studies suggest structural similarities with picrotoxin including a lateral lipid chain and a hydrophilic membrane, which act as a GABA.

GABA (gamma aminobutyric acid) A and GABA B are receptor antagonists. Therefore, the bilobalides act on the inotropic GABA receptors located on the chlorine channels site as an allosteric negative modulator. The main difference is given by the anticonvulsant effect of bilobalides. This is a positive effect resulting from the inhibition of glutamate, thus leading to the decrease in the synaptic excitation, which represents a neuroprotective effect.

The flavonoids represent a class of phenolic compounds frequently found in plants. Quercetin is often found in a large variety of fruits and vegetables: tea, coffee, and other beans. A large
quantity of flavonoid has a strong inhibition effect on the thyroid peroxidase inhibitor activity. The antioxidant characteristics, as a free radical neutralizer, represent an important property of flavonoids. The antioxidant, antithrombotic, anti-inflammatory, and hypolipidemic effects play a significant role in decreasing the cardiovascular mortality in the context of an increased intake of flavonoids. In fact, flavonoids can constitute a huge nutritional and therapeutic point of interest for modern medicine [34–38].

Based on the data reported in the literature, the peak pair appearing at ca. 200 mV corresponds to rutin (quercetin-3-O-rutinoside— the glycoside formed from the quercetin and the rutinose) and the peak pair observed at cca. 400 mV is related to the presence of the terpenoid compounds ginkgolide and bilobalide [22,39]. The differences observed between the cyclic voltammograms of the pharmaceutical products can be related to the different formulation, different quantity of Ginkgo biloba leaves, and the extraction of active compounds before formulation, etc. However, in both pharmaceutical products, the presence of antioxidant compounds is clearly demonstrated by the intense peaks obtained by cyclic voltammetry.

In Figure 5, it can be observed that the anodic and cathodic peaks were more intense in the case of the pharmaceutical product Tanakan. Therefore, it can be concluded that GinkoPrim Max has less of an antioxidant effect compared to Tanakan, although the quantity of the Ginkgo biloba extract included in the pharmaceutical formulation is greater. According to the product labels, the pharmaceutical product Tanakan contains 40 mg of extract from Ginkgo biloba, while GinkoPrim Max contains 120 mg of extract. The results obtained using cyclic voltammetry can be due to the fact that the extracts are not standardized and the concentration of the biological active compounds is unknown.

The % DPPH Reduction value obtained for Tanakan was 88.23%, and 60.54% for GinkoPrim Max, indicating the good antioxidant properties of both pharmaceutical products. These values are in agreement with the values reported for Ginkgo biloba extracts and other natural products [21,40].

Comparing the results obtained by the DPPH assay and cyclic voltammetry measurement could obtain information about the redox properties and radical scavenging activity induced by electron and hydrogen-transfer [40]. Comparing the currents of the most intense anodic peaks observed in the cyclic voltammograms for Tanakan and for GinkoPrim Max, a significant difference was observed of 17.84 μA for Tanakan and 12.15 μA for GinkoPrim Max. The current values of the anodic peak are in good agreement with the % DPPH reduction values. Therefore, the compounds from the Ginkgo biloba samples detected by both methods are responsible for the antioxidant properties of this valuable natural source.
4. Conclusions

The voltammetric method using the screen-printed electrodes modified with carbon nanotubes is useful for the determination of the antioxidant activity of pharmaceutical products based on *Ginkgo biloba*. This technique offers fast measurements, which can be obtained in a matter of minutes, with low costs. Additionally, it can be used for biological samples or dietary extracts at any pH of interest. Other advantages of this method are its sensitivity and the fact that no chemical reagents are used. Tanakan has a superior antioxidant effect compared to GinkoPrim Max due to the numerous extraction and purification stages applied in order to eliminate the plant fractions responsible for any harmful effects. The OTCs do not undergo as many extraction and purification stages, which can lead to a metabolic and enzymatic competitive effect (Cytochrome P450). For this reason, the efficiency of the active substance drops. Regarding the RX products, by using the purification and extraction methods, the same quantities of final product were obtained, unlike the OTCs. A good correlation between the results of the DPPH assay and cyclic voltammetry was obtained.

Author Contributions: Conceptualization, C.A. and R.O.G.; Writing—original draft preparation, R.O.G.; Writing—review and editing, C.A. and R.O.G.; Supervision, C.A. All authors have read and agreed to the published version of the manuscript.

Funding: The contribution of author Ramona Oana Gunache (Roșca) was supported by the project entitled Excelenţa academică şi valori antreprenoriale—sistem de burse pentru asigurarea oportunităţilor de formare şi dezvoltare a competenţelor antreprenoriale ale doctoranţilor şi post doctoranţilor—ANTREPRENORDOC, contract number 36355/23.05.2019 POCU/380/6/13—SMB code: 123847. The publication fees for this article were supported by “Dunarea de Jos” University of Galati.

Conflicts of Interest: The authors declare no conflict of interest.

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