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Development of Novel Electrochemical Biosensors Based on Horseradish Peroxidase for the Detection of Caffeic Acid

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Abstract: In this study, biosensors based on two types of screen-printed carbon and Prussian blue-carbon electrodes, respectively, modified with peroxidase extracted from horseradish root for the sensitive and selective detection of caffeic acid were developed. The presence of the enzyme in the aqueous extract and the activity of peroxidase was demonstrated by spectrometric methods. The electrochemical technique used for the determination of caffeic acid with the biosensors was the cyclic voltammetry. Calibration of the biosensors towards caffeic acid was carried out in solutions of different concentrations, ranging from 5 to 74 μM. Suitable sensitivities and detection limits for practical applications were obtained, with the more sensitive (0.72 $\mu A \cdot \mu M^{-1}$) one being the biosensor containing Prussian blue as a mediator of the exchange between electrons with a detection limit of 0.9 μM. Caffeic acid was successfully determined and quantified in three food supplements using the Prussian blue-peroxidase-based biosensor. The method used to validate the results obtained with the biosensor in the food supplements was a comparison with the amounts indicated by the producers, with no differences between the results at a 99% confidence level.

Keywords: biosensor; peroxidase; Prussian blue; caffeic acid; cyclic voltammetry; spectrometry

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1. Introduction

Phenolic compounds, natural compounds found mostly in fruits and vegetables, are secondary plant metabolites and contribute to the oxidative stability of biological systems as compounds with antioxidant properties [1–3]. For this reason, they are the subject of numerous scientific research studies on their beneficial effects on human health [3].

One important compound is caffeic acid (3,4-dihydroxycinnamic acid), which belongs to the class of phenolic acids, one of the main classes of phenolic compounds. It is found in high levels in coffee, green tea, olive oil, and wine, and it is known for its antioxidant activity due to its chemical structure (phenolic groups in positions 3 and 4), which allows the transfer of electrons and hydrogen atoms to reduce free radicals that are harmful to the human body [4,5]. Caffeic acid has several benefits for the human body due to its antioxidants and its antibacterial, anti-inflammatory, and immune-modulating compounds [6–9].

Several methods are used for the analysis of caffeic acid in samples of natural origin: Folin–Ciocalteu [10–13], high-performance liquid chromatography (HPLC) [14–17], spectrometric [18–20] methods, and electrochemical methods [21–23].

Electrochemical methods, compared to other analytical methods, have a number of advantages, such as simplicity, ability to measure process kinetics, quantification of analyts in real time, low cost, few reagents being required, short analysis time, and high sensitivity, usually with limits of detection in the range from micromolar to nanomolar [24–26]. However, new biosensors, especially enzyme-based ones, have been developed to increase the sensitivity and especially the selectivity [27–30].

The use of enzymes in making new biosensors is of great interest, and enzyme-rich plant extracts have been used to obtain biosensors with adequate analytical performance

that are useful for the practice. One biological material that can be used to extract an important enzyme, peroxidase, is horseradish root [31,32].

Peroxidase is an enzyme of the oxidoreductase class, which uses hydrogen peroxide as an electron acceptor. In turn, the enzyme accepts the electron made available by caffeic acid, which behaves as an antioxidant agent [33]. This process underlies the possible detection of caffeic acid and other antioxidant compounds based on biosensors containing peroxidase on the receptive element.

Several types of peroxidase-based biosensors have been reported in the literature for the detection of various phenolic compounds. The most important peroxidase-based biosensors used for the detection of some phenolic compounds, the sensitive material, the detection technique, and the substrate (analyte) and detection limits (LOD) are all shown in Table 1.

Table 1. Peroxidase-based biosensors used to detect phenolic compounds.

Sensitive Material	Detection Technique	Analyte	LOD (μM)	Reference
HRP/thiol-modified gold	Amperometry	(+)-Catechin	2.2	[34]
Au/MPA/HRP	Amperometry	Guaiacol	0.8	[35]
SAP/HRP/Au-GN	Cyclic Voltammetry Linear Scanning Voltammetry	Butylated hydroxyanisole Propyl gallate	0.255 0.113	[36]
Poly(Gly)/SiSG/MWCNTs/HRP	Differential pulse voltammetry	Dopamine	0.6	[37]
Con A-HRP	Amperometry	Catechol Phenol o-Cresol	0.6 0.2 0.4	[38]
HRP/DNA	Amperometry	Chlorogenic acid	0.7	[39]
Poly(GMA-co-MTM)/PPy/CNT/HRP	Amperometry	Phenol Hydroquinone Pyrocatechol 2-Aminophenol	0.732 0.336 0.516 0.247	[40]

HRP—horseradish peroxidase; Au/MPA/HRP—gold/mercaptoundecanoic acid/horseradish peroxidase; SAP/HRP/Au-GN—Spiny Au-Pt nanotubes/horseradish peroxidase/Au-graphene; HRP/DNA-horseradish peroxidase/DNA; Con A/HRP—concanavalin A/horseradish peroxidase Poly(Gly)/SiSG/MWCNTs/HRP-DA—poly (Glycine)/silica sol–gel/multiwalled carbon nanotubes/horseradish peroxidase; Poly(GMA-co-MTM)/PPy/CNT/HRP—poly(glycine methacrylate-co-3-thienylmethyl methacrylate)-polypyrrole-carbon nanotube-horseradish peroxidase.

In recent years, as can be seen in Table 1, numerous electrochemical biosensors have been developed for the sensitive detection of phenolic compounds with various chemical structures using commercial peroxidase as a biocatalyst. The main advantages of the enzymatic biosensors for detecting different analysts are the easy fabrication, the reproducibility of the fabrication process, and the good stability when compared to the affinity biosensors, which have limited application in the analysis of real samples due to the variability of the antibody production process and the short shelf life of the antibodies. However, the disadvantages include high costs and the loss of the biocatalytic properties of the enzyme during the purification process and storage. Therefore, the development of the novel biosensors, such as those fabricated and characterized in this study, based on enzymes directly extracted from the natural sources and screen-printed technology, could feasibly be used in the screening analysis. The use of raw extract without the purification steps could significantly reduce the costs and open new perspectives regarding the use of natural products in the development of biosensors. The commercial enzyme can suffer modifications during purification and dehydration, especially in relation to the tridimensional structure of the protein part, and can partially lose specificity for specific analysts. It can be remarked that in the raw extract, the enzyme is surrounded by the cofactors and other compounds from the biological cells, and, therefore, the biocatalytic properties are well preserved and useful in the electrochemical detection.

The main novelty of this study is the use of horseradish root extract containing peroxidase for the construction of new biosensors using screen-printed electrodes as support electrodes and Prussian blue as an electron exchange mediator for the detection of caffeic acid. Based on our knowledge, it is the first time a biosensor based on Prussian blue and peroxidase has been developed for the detection of caffeic acid. Spectrometric methods will be used on horseradish root extract to identify and characterize enzymatic activity. The biosensors will be developed, characterized, and successfully applied for the qualitative and quantitative determination of caffeic acid in standard solutions and real samples.

2. Materials and Methods

2.1. Equipment

The ultraviolet-visible (UV-Vis) spectrophotometer RayLeigh UV 1601 (Beijing Beifen–Ruili Analytical Instrument, Beijing, China) was used for spectrophotometric analysis, and the Bruker Alpha-E FTIR spectrometer (BrukerOptik GmbH, Ettlingen, Germany) was used for infrared (IR) analysis.

An EG&G potentiostat/galvanostat, Model 263 (Princeton Applied Research, Oak Ridge, TN, USA), an electrochemical cell with three electrodes, an Ag/AgCl reference electrode, a platinum wire (as the auxiliary electrode), and a peroxidase-based biosensor (as the working electrode) were used to record the biosensor responses. Cyclic voltammetry, which is based on the measurement of the current when cyclically scanning the potential in a certain optimal potential range, was the method used for the detection and quantification of caffeic acid [41].

An Elmasonic ultrasonic bath (Carl Roth GmbH, Karlsruhe, Germany) was used for enzyme extraction and the homogenization of solutions.

2.2. Reagents and Solutions

An aqueous extract of horseradish root, 10^{-3} M caffeic acid stock solution in 0.01 M phosphate buffer solution (PBS) of pH 7, 0.01 M PBS of pH 7, 3% hydrogen peroxide solution, glutaraldehyde, gallic acid, caffeic acid, catechol, and catechin were all used in this study. The chemicals were purchased from Sigma–Aldrich (St. Louis, MI, USA) and were used because they have analytical purity. Solutions were prepared with ultrapure water obtained using a Millipore ultrapurification system (Millipore, Bedford, MA, USA). Horseradish root extract was obtained from 11 g of ground horseradish root, which was mixed with 20 mL ultrapure water, and the liquid phase was separated by filtration.

2.3. Spectrophotometric Methods

In a quartz cuvette with a 10 mm optical pathway, 5 µL of horseradish extract was added, made up to 3 mL with PBS pH 7, and it was analyzed spectrophotometrically in the UV range.

The enzyme was also identified by IR spectroscopy, with both the aqueous extract and the raw horseradish root analyzed.

2.4. Study of Enzymatic Activity

Peroxidase activity was studied spectrophotometrically using the peroxidase biocatalyzed gallic acid oxidation reaction. The sample, prepared from 4.995 mL of gallic acid solution 0.3% with 10^{-2} M hydrogen peroxide in 10^{-2} M PBS pH 7 and 5 µL horseradish extract, was analyzed 10 min after the addition of the enzyme to the solution in the range of 300 to 400 nm.

2.5. Biosensors Development

For the analysis of the antioxidant activity of caffeic acid and its quantification, two biosensors were developed from two screen-printed electrodes, one carbon based (SPCE) and one Prussian blue-modified carbon (SPCE/PB) acquired from Metrohm Dropsens (Llanera, Spain). The horseradish peroxidase (HRP) enzyme was deposited on the two

types of electrodes by the drop-and-dry technique, adding exact volumes of extract. Then, 10 µL of horseradish extract was deposited on both screen-printed electrodes. After the evaporation of the water, cross-linking of the enzyme was performed by exposure to glutaraldehyde vapor for 3 min. Two biosensors, SPCE/HRP and SPCE/PB/HRP, were obtained and these devices were tested for the detection of caffeic acid.

2.6. Electrochemical Measurements

For the electrochemical analysis, cyclic voltammograms were recorded using the Echem version 4.30 software (Princeton Applied Research, Oak Ridge, TN, USA) for the control and data acquisition in the potential range from −0.4 V to +1.3 V and with a scan rate of 0.1 V-s^{-1}. The reference electrode was Ag/AgCl, the counter electrode was a platinum wire, and the biosensors were the working electrodes. The biosensors were immersed in solutions of different concentrations of caffeic acid, obtaining cyclic voltammograms specific to each concentration. Based on the peak currents and the concentrations of the solutions, regression linear models for caffeic acid were developed.

2.7. Food Supplements Analysis

The three food supplements analyzed were Telom-R Diab (DVR Pharm), Calmogen Plant Somn (Omega Pharma), and DVR-Stem Glycemo (DVR Pharm), and this was done by means of the SPCE/PB/HRP biosensor. The anodic peak currents registered with the SPCE/PB/HRP biosensor at 1.100 V were used for the quantification of caffeic acid in the samples by interpolation with the calibration equation.

3. Results

3.1. Identification of Peroxidase by Spectrometric Methods

The Fourier Transform infrared (FTIR) method was used to identify the peroxidase in horseradish root using the attenuated total reflection (ATR) technique as the sample exposure method. A small part of the horseradish root was sampled and the spectrum was recorded using the air as background (Figure S1).

The same procedure was used to obtain the spectrum of the horseradish extract, but ultrapure water was used as a background (Figure S2).

The IR spectra registered in the 4000–500 cm^{-1} range for horseradish root (Figure 1) and horseradish root extract (Figure S2) show peaks characteristics of the functional groups of peroxidase: 3300–2900 cm^{-1} and 1440–1390 cm^{-1} for the OH group; 1650–1580 cm^{-1} for the N-H bond in the α-helix structure; 1250–1020 cm^{-1} for the C-N bond; 999/996 cm^{-1} for the C=C bond; and 924/927 cm^{-1} for Fe-OH group [42].

UV spectrophotometry was also used to identify the enzyme, analyzing the horseradish extract. To record the spectrum, a 5 µL extract was taken and made up to 3 mL with PBS solution pH 7 (Figure S3).

Figure S3 shows the spectrum of the HRP extract solution in the UV range, with a peak at 260 nm wavelength with an absorbance of 0.667, a peak related to peroxidase from the solution [43].

Therefore, the main features of the peroxidase enzyme molecule were evidenced in the raw material and in the extract obtained from horseradish root by UV and IR spectrometric methods, in agreement with the results reported in the literature [42,43].

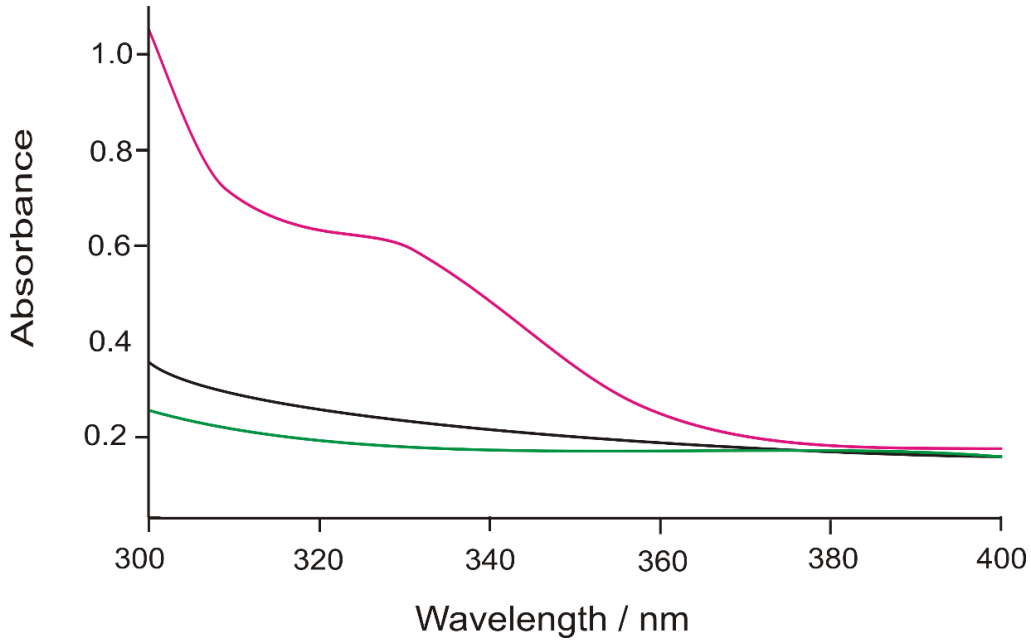

Figure 1. Spectrum of the gallic acid sample with 3% hydrogen peroxide in PBS pH 7 and 5 µL of horseradish extract (red line); spectrum of the 3% hydrogen peroxide in PBS pH 7 and 5 µL of horseradish extract (black line); and spectrum of 5 µL of horseradish extract in PBS pH 7 (green line).

3.2. Study of Enzymatic Activity

The enzymatic activity of peroxidase was analyzed by spectrophotometry in the 300–400 nm range. For the solution prepared from 4.995 mL 0.3% gallic acid with 10^{-2} M hydrogen peroxide in 0.01 M PBS pH 7 and 5 µL horseradish extract, a maximum absorbance of 0.61 was obtained at a 335 nm wavelength (Figure 1—red line), specific for the oxidation compounds of gallic acid. In the absence of the enzymatic substrate, gallic acid, the control solution did not shown any absorbance peak in the same wavelength range (Figure 1—black line). The spectrum of the horseradish extract did not show peaks in the 300–400 nm range (green line).

To determine the optimum amount of enzyme extract optimal for gallic acid oxidation from the aqueous solution of a concentration of 0.3% and 10^{-2} M hydrogen peroxide, varying volumes of extract were added to the same volume of gallic acid solution and kept for 10 min at room temperature. The absorbance was then determined at 335 nm and it was observed that the absorbance reached the maximum value when 10 µL of horseradish extract was used. The results obtained are presented in Figure S4. Therefore, this amount of extract was used for the development of biosensors.

3.3. Characterization of Biosensors by Cyclic Voltammetry

Considering the characteristics of the sensors and the spectrophotometric results regarding the activity of the enzyme, it was demonstrated that the volume of 10 µL is the optimal volume of horseradish extract that can be deposited on the two screen-printed electrodes in order to obtain biosensors with improved electroanalytical characteristics, which could be used in laboratory practice.

Both the SPCE/HRP biosensor and the SPCE/PB/HRP biosensor were firstly immersed in PBS pH 7 and 10^{-2} M hydrogen peroxide solution to obtain stable responses (Figures 2 and 3) and the characteristic biosensors responses in the support electrolyte solution (Figures 2 and 3) were registered at the optimal scan rate of 0.1 $V \cdot s^{-1}$.

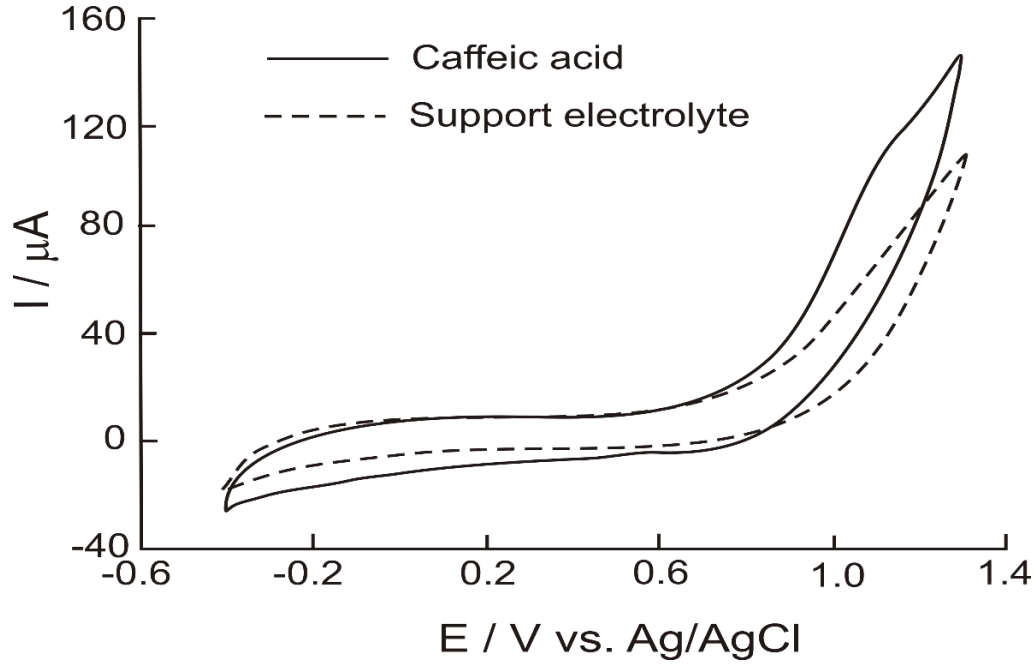

Figure 2. Cyclic voltammogram of SPCE/HRP biosensor immersed in electrolyte support solution (10^{-2} M PBS pH 7–10^{-2} M hydrogen peroxide), (dashed line); cyclic voltammogram of SPCE/HRP biosensor immersed in 30 µM caffeic acid solution in electrolyte support solution (solid line).

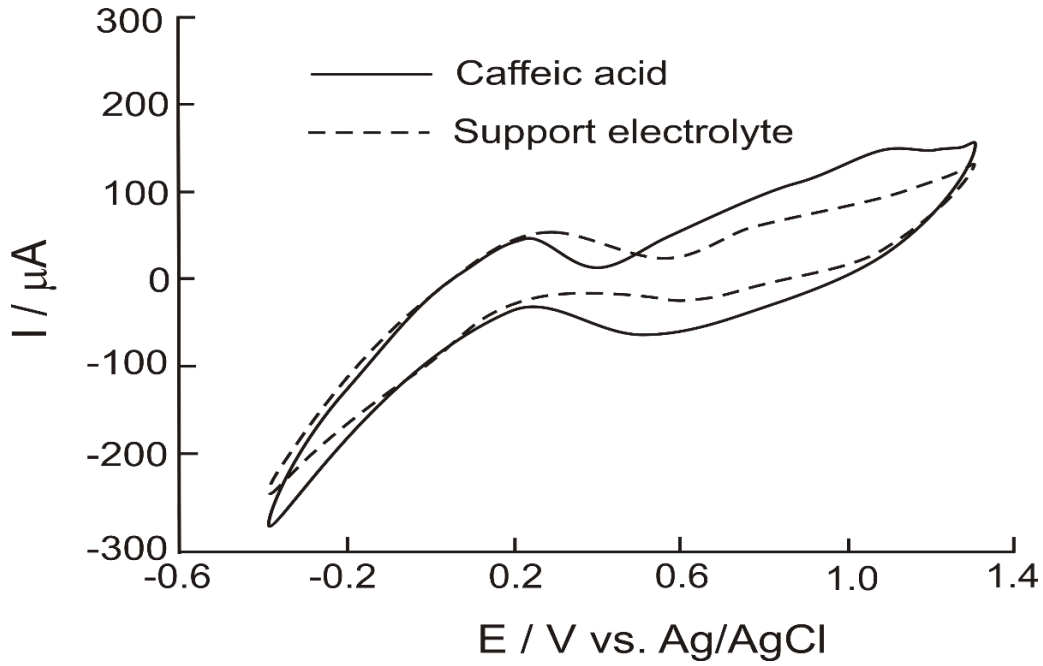

Figure 3. Cyclic voltammogram of SPCE/PB/HRP biosensor immersed in electrolyte support solution (10^{-2} M PBS pH 7–10^{-2} M hydrogen peroxide), (dashed line); cyclic voltammogram of SPCE/PB/HRP biosensor immersed in 30 µM caffeic acid solution in electrolyte support solution (solid line).

The cyclic voltammogram of the SPCE/HRP biosensor shows no peaks in the analyzed solution, while two pairs of redox peaks are observed for SPCE/PB/HRP. It is observed that the SPCE/PB/HRP biosensor is more sensitive than the other biosensor due to Prussian blue, which is electroactive, and the electrochemical processes taking place at the biosensor surface are shown in Equations (1) and (2) [44–46].

$$\underset{\text{Prussian blue}}{Fe^{III}[Fe^{II}(CN)_6]} + 4e^- + 4K^+ \leftrightarrow \underset{\text{Prussian white}}{K_2Fe^{II}[Fe^{II}(CN)_6]} \quad (1)$$

$$\underset{\text{Prussian blue}}{Fe^{III}[Fe^{II}(CN)_6]} - 3e^- + 3Cl^- \leftrightarrow \underset{\text{Berlin green}}{Fe^{III}[Fe^{III}(CN)_6 \cdot Cl]_3} \quad (2)$$

The SPCE/HRP and SPCE/PB/HRP biosensors were immersed in solutions with the same concentrations of caffeic acid, 30 μM and the cyclic voltammograms obtained are shown in Figures 2 and 3 (solid lines). As can be observed in Figures 2 and 3, in the cyclic voltammograms, anodic and cathodic peaks related to the redox processes of caffeic acid in the solution to be analyzed were observed.

In the case of the SPCE/HRP biosensor, immersed in 30 μM caffeic acid solution, currents of −20.98 μA at potential of −0.200 V for the cathodic peak and 92.27 μA at potential of 1.100 V for the anodic peak were obtained (Figure 2).

In the case of the SPCE/PB/HRP biosensor, immersed in 30 μM caffeic acid solution, current peaks of −41.78 μA at the −0.100 V and −22.4 μA at 0.550 V for the cathodic peaks, as well as 147.5 μA at the 1.100 V and 20.2 μA at 0.190 V for the anodic peaks were observed (Figure 3).

The detection mechanism of the SPCE/PB/HRP is presented in Figure 4.

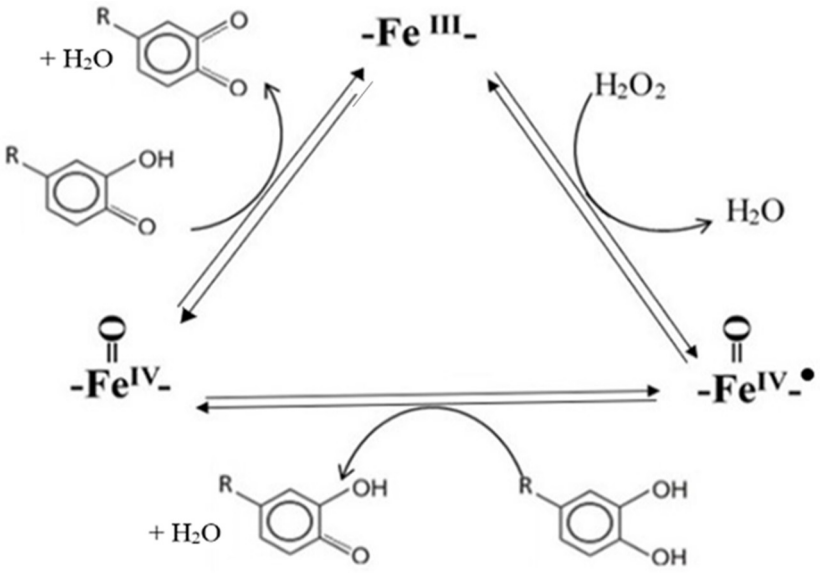

Figure 4. The detection principle of the Prussian blue-peroxidase based biosensor towards caffeic acid in the presence of hydrogen peroxide. R is -CH=CH-COOH.

Regarding the oxidation reduction processes of caffeic acid at the sensitive element of the biosensor, it passes into its o-quinone derivative by losing two electrons and two protons and peroxidase (oxidized by hydrogen peroxide), and it reduced caffeic to its initial form by accepting two protons and two electrons [47].

Since higher current values were obtained, the SPCE/PB/HRP biosensor is found to be more sensitive than the SPCE/HRP biosensor, due to the Prussian blue facilitating electron exchange between the redox reaction and the electrode surface [48].

3.4. Influence of the Caffeic Acid on the Biosensors Responses

For the calibration of the biosensors, the signals were recorded in solution of caffeic acid with different concentrations.

Figure 5a depicts the overlapped cyclic voltammograms of the SPCE/HRP biosensor immersed in caffeic acid solution concentrations ranging from 5 μM to 74 μM (not all are shown).

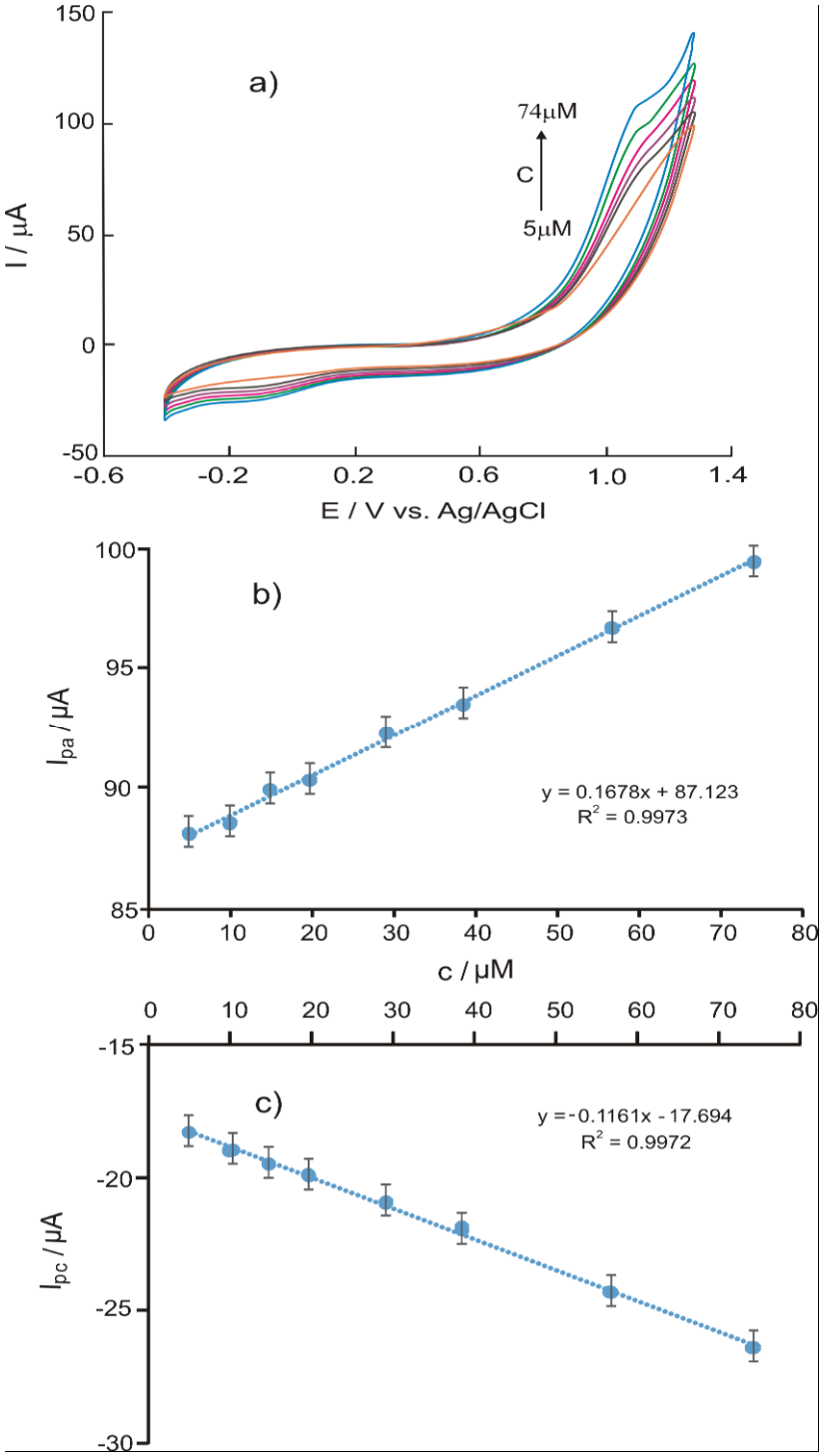

Figure 5. (**a**) Overlapping cyclic voltammograms of the SPCE/HRP biosensor, immersed in different solutions with variable concentrations of caffeic acid (electrolyte support was 10^{-2} M PBS pH 7–10^{-2} M hydrogen peroxide solution); (**b**) plot of linear calibration of the SPCE/HRP biosensor for caffeic acid based on anodic peak currents; and (**c**) plot of linear calibration of the SPCE/HRP biosensor for caffeic acid based on cathodic peak currents.

The response of the SPCE/HRP biosensor was recorded for each concentration of caffeic acid, resulting in current values directly proportional to the substrate concentration for both the cathodic peak (at −0.100 V) and the anodic peak (at 1.100 V).

After recording the response, linear regression models of the SPCE/HRP biosensor (for anodic peak, Figure 5a; and for cathodic peak, Figure 5b) were obtained for the detection of caffeic acid from the dependence between the intensity of the peak current and caffeic acid concentration.

From the slope of the dependence between the current and the concentration of caffeic acid in the solution, the detection and quantification limits of the biosensor were calculated. The standard deviation for the blank sample was calculated from seven replicates of cyclic voltammograms recorded in an electrolyte solution containing no caffeic acid. The sensitivity of the SPCE/HRP biosensor based on anodic peak variation is 0.17 μA·μM, indicating an adequate performance for the detection of caffeic acid.

For the anodic peak, the limit of detection (LOD) is 3.86 μM and the limit of quantification (LOQ) is 12.87 μM, calculated according to Equations (3) and (4).

$$\text{LOD} = 3 \times \sigma/\text{m} \quad (3)$$

$$\text{LOQ} = 10 \times \sigma/\text{m} \quad (4)$$

where σ is the standard deviation of the biosensor signal in the blank sample and m is the slope of the calibration linear equation [49].

For the cathodic peak, the limit of detection (LOD) is 5.58 μM and the limit of quantification (LOQ) is 18.60 μM

Figure 6a shows the overlaid cyclic voltammograms of the SPCE/PB/HRP biosensor immersed in 10^{-2} M PBS pH 7–10^{-2} M hydrogen peroxide solution and caffeic acid solutions in a concentration range from 5 μM to 74 μM (not all are shown).

The response of the SPCE/PB/HRP biosensor in caffeic acid solutions of different concentrations was recorded, resulting in current values directly proportional to the substrate concentration for both the cathodic peak (at E of −0.100 V) and the anodic peak (at E of 1.100 V).

From the cyclic voltammograms, calibration linear models of the SPCE/PB/HRP biosensor (for both peaks—Figure 6b,c) were made for the detection of caffeic acid, taking into account the current intensities as a function of the concentration of caffeic acid in the solution, and the detection and quantification limits of the biosensor were calculated. The sensitivity of the SPCE/PB/HRP biosensor taking into account the anodic peak increment when the concentration increases is 0.72 μA·μM^{-1}. This value is four times higher compared with the sensitivity obtained for the SPCE/HRP biosensor. This fact demonstrates the importance of the PB from the sensitive layer as the electron mediator.

For the anodic peak, the limit of detection (LOD) is 0.90 μM and the limit of quantification (LOQ) is 3.00 μM. For the cathodic peak, the limit of detection (LOD) is 1.96 μM, and the limit of quantification (LOQ) is 6.53 μM.

From the data presented, it appears that the SPCE/PB/HRP biosensor has the highest sensitivity when the anodic peak current is used as an input parameter. The detection limit of 0.90 μM is better than the value obtained for other biosensors (not containing the electrons mediator) developed in this study.

The SPCE/PB/HRP biosensor sensitive characteristics in comparison with other biosensors reported in the literature when caffeic acid is detected are presented in Table 2.

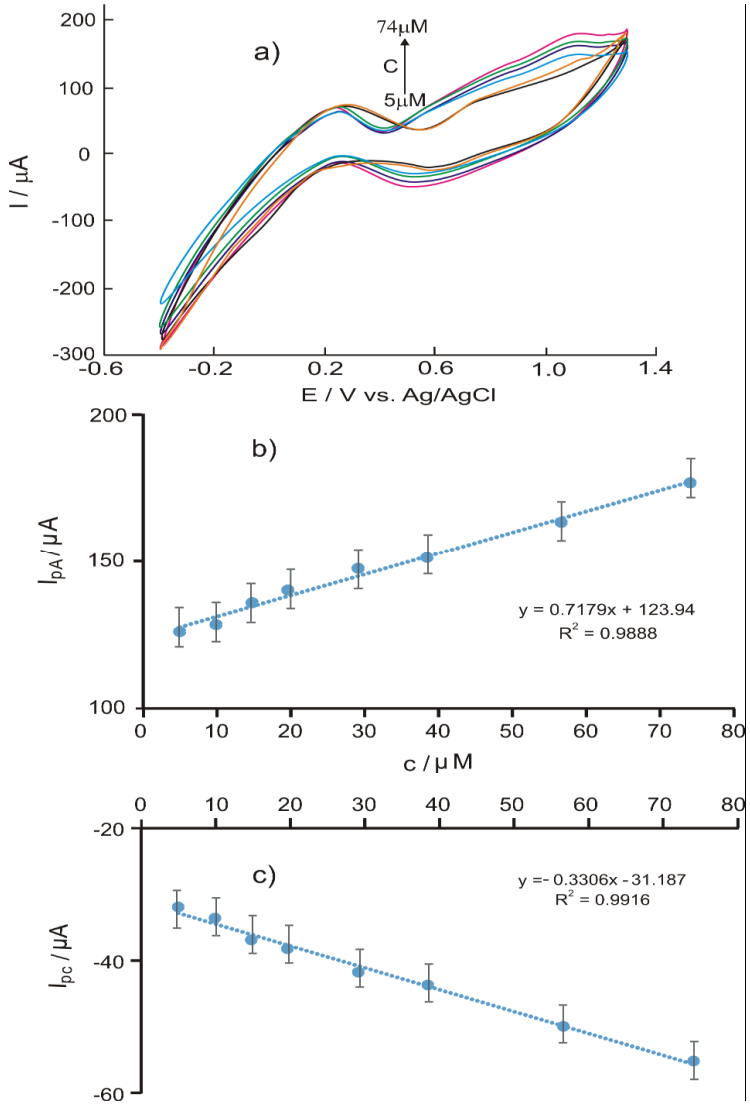

Figure 6. (**a**) Overlapping cyclic voltammograms of SPCE/PB/HRP biosensor, immersed in different solutions with variable concentrations of caffeic acid (electrolyte support was 10^{-2} M PBS pH 7–10^{-2} M hydrogen peroxide solution); (**b**) plot of linear calibration of the SPCE/PB/HRP biosensor for caffeic acid based on anodic peak currents; and (**c**) plot of linear calibration of the SPCE/PB/HRP biosensor for caffeic acid based on cathodic peak currents.

Table 2. Enzymatic biosensors used to detect caffeic acid.

Sensitive Material	Detection Technique	Linearity Range (μM)	Limit of Detection (μM)	Reference
PEDOT-Tyr	CV	10–300	4.33	[50]
MoS_2-GQDs-TvL	CV	0.38–10 10–100	0.32	[51]
GNP@MnO_2/Lac	Amperometry	5–320	1.9	[52]
GCE/TvL	Amperometry	4–55	4	[53]
AgCl/Ag-Pt/polyethersulfone membrane/Laccase	Amperometry	2–14	1	[54]
Polyethersulfone membranes/Laccase	Amperometry	5–35	0.88	[55]
PB/HRP	CV	5–74	0.9	This study

GNP—graphene nanoplatelets; MnO_2—manganese(IV)-oxide; Lac-Laccase; PEDOT—Poly(3,4-ethylenedioxythiophene); Tyr—Tyrosinase; MoS_2-GQDs—Molybdenum disulphide and graphene quantum dots; TvL—*Trametes versicolor* Laccase; CV—cyclic voltammetry; PB—Prussian blue; HRP—horseradish peroxidase.

As can be observed in Table 2, the SPCE/PB/HRP biosensor possesses better or comparable performance characteristics with other biosensors reported in the literature. However, the advantages of this type of biosensor are ease of preparation, use of a natural extract in which the enzyme retains its biocatalytic activity, high sensitivity, and low cost.

Therefore, the SPCE/PB/HRP biosensor has enough good performance characteristics to be used in practice for the detection of caffeic acid in real samples.

3.5. Interference Studies

For the estimation of the interferences related to different species on the detection of caffeic acid at the 20 μM level, different interference studies on the SPCE/PB/HRP biosensor were set up by cyclic voltammetry. The compounds used in these studies were different phenolic compounds with a similar chemical structure to caffeic acid, namely, catechol, catechin, and gallic acid. The concentration of interfering phenolic compounds in the solutions was 20 μM.

The response of the SPCE/PB/HRP biosensor immersed in 20 μM caffeic acid and 20 μM caffeic acid solution in the presence of electrolyte support (solid line) and the response in the electrolyte support (dashed line) are shown in the Figure 7.

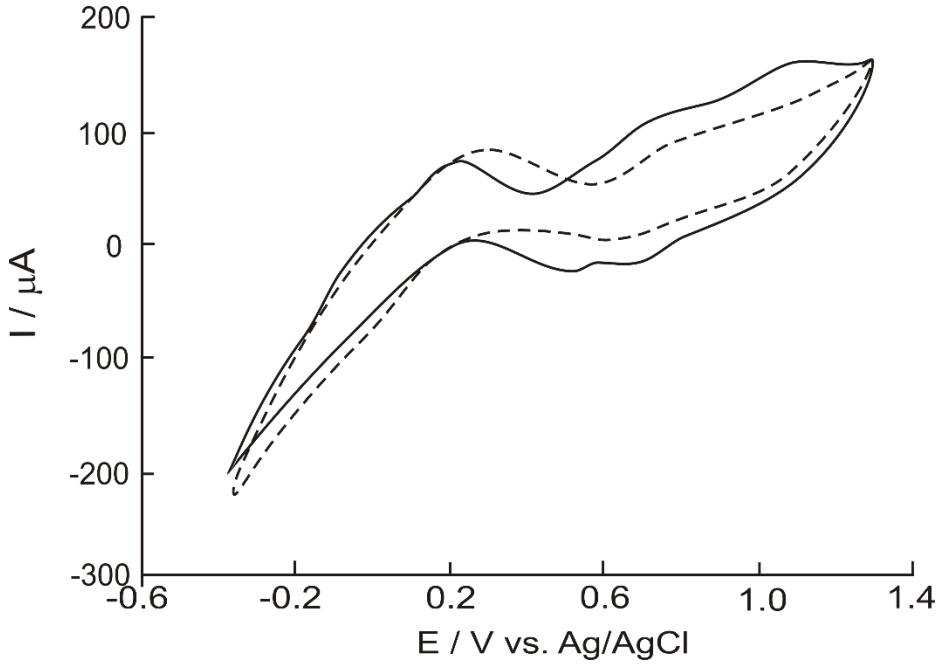

Figure 7. Cyclic voltammogram of SPCE/PB/HRP biosensor immersed in 20 μM caffeic acid and 20 μM caffeic acid solution in 10^{-2} M PBS pH 7–10^{-2} M hydrogen peroxide solution (solid line); cyclic voltammogram of SPCE/PB/HRP biosensor immersed in 10^{-2} M PBS pH 7–10^{-2} M hydrogen peroxide solution (dashed line).

As can be observed, the peak corresponding to caffeic acid is appearing at 1.105 V and the current is 141.3 μA—values very close to those obtained in the solution containing only caffeic acid. Two peaks related to catechol were observed: one anodic (at 0.700 V) and one cathodic (0.680 V). However, the SPCE/PB/HRP biosensor is able to accurately detect the caffeic acid in the presence of catechol.

Other experimental results obtained in the interference studies are included in Table 3. As can be seen, all the phenolic compounds studied had reduced interference in the determination of caffeic acid. The biosensor showed low sensitivity to the various interfering compounds found in multicomponent solutions and high selectivity for the caffeic acid.

Table 3. Interference study results on the response of biosensor SPCE/PB/HRP immersed in 20 μM of caffeic acid.

Caffeic Acid 20 μM + Interfering Compound 20 μM	Potential (V)	Potential Change (%)	Current (μA)	Current Change (%)
Caffeic acid	1.100	-	140.2	-
Caffeic acid + catechol	1.105	0.45	141.3	0.78
Caffeic acid + catechin	1.092	0.72	138.7	1.07
Caffeic acid + gallic acid	1.110	0.91	143.5	2.35

This study demonstrated that the biosensor could be used for the detection of caffeic acid in real samples in the presence of interfering compounds, even at high concentrations. These results also confirmed that the peak currents and potentials related to the presence of caffeic acid are slightly influenced by the other phenolic compounds from the analyzed solution.

3.6. Stability of the Biosensor Response

Stability is one important feature for the development of the feasible biosensors. In this study, the stability of the biosensor was studied in two ways. The repeatability of 50 successive measurements in 20 μM solution was studied. The anodic current corresponding to oxidation of caffeic acid decreased by 4.54% after 50 successive measurements.

The storage stability was evaluated for 30 days. Between uses, the biosensor was stored at 4 °C. After 30 days of storage, the biosensor response (anodic peak current) decreased by 10.5%.

Therefore, the biosensor has enough good stability to be used in laboratory practice.

3.7. Quantification of Caffeic Acid in Food Supplements

The SPCE/PB/HRP biosensor, the biosensor with the best performance characteristics developed in this study, was used to quantify caffeic acid in three food supplements. Cyclic voltammograms recorded with the SPCE/PB/HRP biosensor in the solution of these products show a pair of redox peaks related to the presence of caffeic acid present in the samples to be analyzed, similar to those observed in pure caffeic acid solutions.

The amounts of caffeic acid in the food supplements were calculated from the calibration equations for the biosensor (SPCE/PB/HRP). All quantification experiments were performed in triplicate and the results obtained are reported as averages.

For the voltammetric method, the current corresponding to the specific detection potential for caffeic acid at 1.100 V was taken into account.

For the calculation of the values reported in Table 4, the dilutions and the amount of food supplements used in the analysis were taken into account. Manufacturers indicate that caffeic acid is present in food supplements, specifying the amount in some cases. The results, expressed in mg of caffeic acid per capsule, are shown in Table 4.

Table 4. Caffeic acid content in food supplements.

Food Supplement	Caffeic Acid Content (%)	
	Biosensor	Declared Content by Producer (%)
Telom-R Diab	2.54 ± 0.08	2.5
Calmogen Plant Somn	1.20 ± 0.002	-
DVR-Stem Glycemo	2.47 ± 0.06	2.5

The values of caffeic acid content obtained with the biosensors are close and the analysis of variance (ANOVA) showed that there is no significant difference between the values at a 99% confidence level (p = 0.002), assuming equals variances. This result demonstrates that the voltammetric method is a sensitive method and has very good accuracy for the determination of caffeic acid in food supplements.

4. Conclusions

A series of voltammetric biosensors based on screen-printed carbon and Prussian blue modified carbon electrodes to which horseradish-extracted peroxidase has been added have been successfully used in the analysis of caffeic acid in nutraceuticals. UV and IR spectrometric analysis demonstrated the presence of peroxidase in the aqueous extract of horseradish root and its enzymatic activity. By the immobilization of the enzyme by cross-linking on two types of screen-printed electrodes, biosensors were obtained that revealed the redox properties of caffeic acid. Among the biosensors studied, the one based on carbon, Prussian blue, and peroxidase has excellent sensitivity for the detection of caffeic acid when cyclic voltammetry is used as a detection method. Limits of detection and limits of quantification in the 10^{-6} M range have been obtained, making it possible to use the biosensors for the detection of caffeic acid from various products of interest. The electroanalytical method has been successfully applied for the determination of caffeic acid content in food supplement products. The advantages of this method are that the sample can be analyzed directly after dissolution, and the equipment is simple, portable, and highly sensitive. This electroanalytical method can be used as a screening method to determine the caffeic acid content in food, nutraceuticals, or pharmaceutical products.

The biosensors based on raw extracts containing the enzyme together with the use of nanomaterials could be a solution for the development of cheap, easy to use, and selective novel systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13042526/s1, Figure S1: FTIR spectrum of raw horseradish root. The background was the air; Figure S2: FTIR spectrum of horseradish root extract. The background was the ultrapure water; Figure S3: UV spectrum of the horseradish root extract. Figure S4: Dependence of the absorbance determined at 335 nm towards the adding of different volumes of horseradish extract in a solution containing 0.3% gallic acid with 10^{-2} M hydrogen peroxide in 0.01 M PBS pH 7. Final volume was 5mL for all samples.

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