Laccase Carbon Paste Based Biosensors for Antioxidant Capacity. The Effect of Different Modifiers

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The natural antioxidants of food products have great influence on human health. Indeed, the protective effects against cardiovascular diseases, cancer and ageing have been attributed to their polyphenol content. In turn, the remarkable antioxidant activity of polyphenols can be easily estimated by polyphenol oxidases based biosensors. The aim of this work was check the effect of modifiers on the improvement of biosensors concerning to performance, in order to express the antioxidant activity and total polyphenol content of complex natural samples such as coffee beverages. The biosensors were constructed by occlusion of crude extract of *Pycnoporus sanguineus* containing laccase, in different carbon paste based composites. The electrochemical properties of the resulting composites, namely Carbon Paste-Laccase (CPL), CPL modified with Glutaraldeide (CPL-Glu), Bovine Serum Albumin (CPL-BSA), Chitosan (CPL-Ch), Silica (CPL-SiO), Titanium dioxide (CPL-TiO), DNA (CPL-DNA), Carbon Nanotubes (CPL-CN), non-activated Carbon Nanotubes (CPL-nCN) and activated Carbon Nanotubes plus DNA (CPL-DNA:CN) were evaluated by voltammetry in different experimental conditions. In order, the effect of electrolyte composition, pH, scan rate, pulse amplitude, response time and concentration range were carefully evaluated, whereas rutin and/or cathecol were employed as standard solutions. The highest sensitivity level and lowest detection limit were obtained for CPL-DNA:CN. In addition, the biosensor demonstrated suitable stability over 10 days and good repeatability (RSD < 5%). CPL-DNA:CN was used for the determination of total phenol content of coffee samples, expressed as Gallic Acid equivalents (GAEq) and the results were compared with those from the DPPH and Folin–Ciocalteu spectrophotometric methods. Further, the inherent sensitivity of electroanalytical methods, the amperometric biosensor exhibits some interesting advantages such as simplicity due to the minimal sample preparation and leading to rapidity of antioxidant capacity assay.

1. INTRODUCTION

In analytical chemistry the problem of selectivity, particularly at low analyte concentrations and in the presence of interfering substances is of paramount importance. Among the strategies to overcome these problems emerges the biosensor technology. Biosensors are analytical devices which tightly combine bio recognition elements and physical transducers for detection of the target compounds. Hence, owing to their remarkable practicality and versatility they are already useful analytical tools, applicable in clinical, pharmaceutical, environmental and food analysis, among other fields [1].

In turn, despite the high technological input, there is a wide gap between research and market place [2]. Truly, the great majority of biosensors is only just starting to move from the proof-of-concept stage to field-testing, mass production and commercialization. This "state of art" is mainly due to stability issues and quality assurance [3]. Such sensors can be classified accordingly to the biological recognizing agent and/or the type of transducer employed.

In enzyme-based biosensors, the biological element is the enzyme which reacts selectively with its substrate. The response of an enzymatic biosensor against a selected analyte (subtract) is mainly determined by the concentration of the biochemically generated product at sensor surface. According to the principle of analysis, enzymatic biosensors can be based on: a) catalytic transformation of the analyte; b) specific inhibition of enzyme activity by the analyte and c) modification of enzyme activity by an analyte which acts as a modulator or cofactor [4].

On the other hand, the transducer system might be sensitive to subtract (analyte) or to product. There is a great variety of transducing devices, which combined to the great diversity of materials offer uncountable biosensor design possibilities for an uncountable number of compounds [1].

The efficiency of biosensors is mainly related to the optimal coupling of enzyme to the transducer. Thus, many techniques of immobilization have been proposed, *i.e.* entrapment in polymeric matrices, physico-chemical adsorption or covalent bonding at solid surface and occlusion in nanocomposites [5-8].

Laccase-based enzyme electrodes are commonly designed for the selective determination of phenolic compounds, in which the relevance is figured by organic pollutants and natural antioxidants. Hence, these devices can be applied on the evaluation of total phenol composition, antioxidant capacity, as well as, for tentative determinations of specific "catechol-like" major markers [9, 10]. Their functioning is based on the biochemical oxidation followed by electrochemical reduction, which is easily detected by amperometric transducers [11].

An easy and efficient way to produce an amperometric biosensor is the immobilization of enzyme by occlusion in composites. A mixture of carbon (graphite) powder and an agglutinating liquid is a popular composite material used for the preparation of various modified electrodes [5,12]. Carbon paste allows an intimate contact between biocomponent and sensing sites permitting a fast electron transfer. Aside the versatility in which various modifiers can be incorporated and reasonable stability, the main advantage of carbon paste electrodes is the good reproducibility achieved by the easy renewal of the surface. Carbon paste based biosensors are usually prepared by first mixing the biocomponent

and graphite powder. Then, the resulting powder is mixed with mineral oil (e.g. paraffin or silicone). The final paste is filled into a cylindrical cartridge [5].

Carbon paste electrodes can be modified with a number of inorganic and organic materials, in order to improve the electrical properties [13-17]. Hence, a number of papers about carbon paste laccase-based biosensors have been published [7, 8, 12, 18-21]. Nevertheless, concerned to the effect of modifiers materials on the biosensor performance, an accurate comparison can be only achieved by intra assay experiments. Therefore, the main aim of this work was to evaluate the effect of different materials usually employed in carbon paste laccase-based biosensors.

The electrochemical properties of the resulting composites, namely Carbon Paste-Laccase (CPL) modified with Bovine Serum Albumin (CPL-BSA), Chitosan (CPL-Ch), Silica (CPL-SiO), Titanium dioxide (CPL-TiO), DNA (CPL-DNA), Carbon Nanotubes (CPL-CN), non-activated Carbon Nanotubes (CPL-nCN) and activated Carbon Nanotubes plus DNA (CPL-DCN:CN) were evaluated in different electrolyte conditions by voltammetric techniques. The chosen target was rutin, whereas the biosensor with best performance was applied on the evaluation of antioxidant capacity and total phenol determination in coffee samples.

2. MATERIALS AND METHODS

2.1 Chemicals and Samples

All chemicals presented analytical grade, all solutions were prepared by using double distilled water (conductivity $\leq 0.1 \mu Scm^{-1}$).

2.1.1 Samples

Different commercial trademarks of roasted coffee samples herein used as real targets for application studies were purchased from local markets of Goiania-GO/Brazil.

2.1.1.1 Preparation of the coffee samples

The coffee extracts were prepared by infusion submitting two grams (2 g) of each sample in 25 mL of hot water (100 ºC) by 10 minutes under stirring. Afterwards the coffee extracts were cooled till room temperature and filtered with 0.45 μm filter paper. The resulting solution was subsequently diluted in suitable analytical grade solvents according to the experiment in order to obtain the assay solutions. For the electrochemical measurements, 250 μL of the resulting solution were diluted in 4 mL of the electrolyte solution, *i.e.* pH 5.0 0.1 M phosphate buffer [10].

2.1.2 Phenolic standards

Cathecol, gallic acid, rutin, caffeic acid, chlorogenic acid, phenol and chlorophenol were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2.1.3 Modifiers and production of the enzymatic crude extract

Graphite powder, multi wall carbon nanotubes (MWCNT), Bovine Serum Albumin (BSA), Chitosan, and DNA were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA), Silica, Titanium dioxide and Mineral Oil were purchased from Merck (Darmstadt, Germany). The MWCNT were activated by chemical oxidation in boiling Nitric acid 50%.

The laccase crude extract (2019 $U.L^{-1}$) was obtained by submerged fermentation using five disks (7 mm in diameter) of the *Pycnoporus sanguineus* (CCT-4518) in growth medium containing 12.8 $g.L^{-1}$ malt extract (HiMedia, Mumbai, India) and 0.005 $g.L^{-1}$ $CuSO_4.H_2O$ (Cromoline, São Paulo, Brazil) 50 $mg.L^{-1}$, 2,5-xylidine (Sigma Aldrich Chemical Co., St. Louis, MO, USA) was used as inducer of laccase. The fermentation process occurred under agitation of 150 rpm, at 28ºC, 72h. The mycelium was separate by filtration and the crude extract used as source of laccase [22-24].

2.2 Biosensor preparation

The immobilization was carried out by occlusion in different carbon powder composites. Briefly, 250 µL of laccase crude extract were added to graphite powder and their mixtures with the different modifiers and left to dry at room temperature (Table 1). Then, mineral oil was added and rigorously mixed in order to obtain the homogeneous paste.

Table 1. Biosensors Composition

Biosensor	Enzymatic extract (µL)	Graphite powder (mg)	Modifier (%)	Mineral Oil (µL)
CPL	50 to 500	70	-	20
CPL-Glu	250	65	5	20
CPL-BSA	250	69	1	20
CPL-Ch	250	69	1	20
CPL-SiO	250	65	5	20
CPL-TiO	250	65	5	20
CPL-DNA	250	69	1	20
CPL-nCN	250	65	5	20
CPL-CN	250	65	5	20
CPL-DNA:CN	250	64	1:5	20

Carbon Paste Laccase based biosensor (CPL); Glutaraldeide (Glu); Bovine serum albumin (BSA); Chitosane (Ch); Silica (SiO); Titanium dioxide (TiO); Carbon Nanotubes (CN); non-activated CN (nCN).

A suitable portion of the agglutinated paste was taken to fill the cavity 2 mm diameter and 0.5 mm in depth electrode support, originating the CPL like biosensors.

2.4 Electroanalytical assays

Voltammetric experiments were carried out with a potentiostat/galvanostat µAutolabIII® integrated to the GPES 4.9® software, Eco-Chemie, Utrecht, The Netherlands. The measurements were performed in a 5.0 mL one-compartment electrochemical cell, with a three-electrode system consisting of a carbon paste laccase-based biosensor and modifiers (Ø = 2 mm), a Pt wire and the Ag/AgCl/KCl 3M (both purchased from Lab solutions, São Paulo, Brazil), representing the working electrode, the counter electrode and the reference electrode, respectively. The carbon paste was mechanically renewed before each experiment, in order to ensure reproducible results. The experimental conditions for differential pulse voltammetry (DPV) were: pulse amplitude 50 mV, pulse width 0.5 s and scan rate 5 mV.s^{-1}. The experimental conditions for cyclic voltammetry (CV) were: scan rate of 100 mV s^{-1} and scan range from 0 to 1 V. The DP voltammograms were background-subtracted and baseline-corrected, and then all data were analyzed and treated with the software Origin 8®. All experiments were done at room temperature (21 ±1 ºC) in triplicate (n = 3) and the main electrolyte solution used was the phosphate buffer (PBS).

2.5 Spectrophotometric Antioxidant Assays

The absorbance measurements were recorded with a spectrometer Q798U2VS (Quimis Aparelhos Científicos, São Paulo, Brazil).

2.5.1 Folin-Ciocaulteu Total Phenol Assay

For the determination of total polyphenols, the adjusted Folin-Ciocauteu (FC) method was performed. Two milliliters of water and 0.5 mL of Folin Ciocalteu's reagent were mixed with 100 uL of test samples or standard solutions of gallic acid and the resulting mixture was allowed to react for five minutes. Next, 1.5 mL of 20 % sodium carbonate solution was added and then the volume was completed with distilled water to 10 mL. After 1 h of reaction at room temperature, in a dark environment, the absorbance of the solution was measured by spectrophotometry at 765 nm. All determinations were performed in triplicate. The results were expressed in gallic acid equivalents (GAEq), mg of GA in each mL of coffee brew.

2.5.2 DPPH radical scavenging assay

Radical scavenging activity of diluted coffee beverages was measured based on the redox reaction between 1,1diphenyl-2-picrylhydrazyl (DPPH•), a stable and purple radical, and the intrinsic reducing compounds of coffee, leading to generation of yellow colored DPPH, the reduced form The ability of samples to scavenge DPPH• radicals was determined spectrometrically by the method of Blois (1958) [10,19,20]. Briefly, to 2.5 mL of DPPH• ethanolic solution (0.1 mM) an aliquot of 0.5 mL of ethanol (blank) was added to reach a final volume of 3.0 mL that was repeated for all analytical samples. The reaction solution was incubated for 20 min in the dark at room temperature and measured

at 517 nm, against the blank (A~0.7), whereas ethanol, the solvent used to prepare all solutions, was used in order to adjust the baseline (A = 0.000). Antioxidant activity was expressed as EC_{50}, representing the amount of coffee to produce 50% of decolorization of DPPH• relative to the blank control.

3. RESULTS AND DISCUSSION

The laccase crude extract of the *Pycnoporus sanguineus* was incorporated to solid composites in the range from 50 to 500 µL and the electrochemical performance of the electrodes was evaluated in a broad pH range (2.0 to 8.0). The best response expressed by higher sensitivity, low capacitive currents and lower response time was achieved for 250 µL in mild acidic pH (5.0 to 6.0). Therefore, these conditions were fixed for all mixed composites, as well as for further studies.

3.1 Electrical Properties

The modifying agents can exert some influence on catalytical and/or electrical properties of modified electrodes. Indeed, the improvement of electrodic properties, such as lower resistivity leads to lower capacitive currents, and has indirect action on the enhancement of sensor sensitivity. Such influence can be monitored by measuring the faradaic electron transfer rate of the reversible ferro/ferricyanide redox system (Figure 1A), and in some extent by observing the profile of blank buffer solution [24]. These statements are well evidenced on Figure 1, where it can be clearly observed how small proportions of different modifiers can change expressively the electrical properties of electrodes.
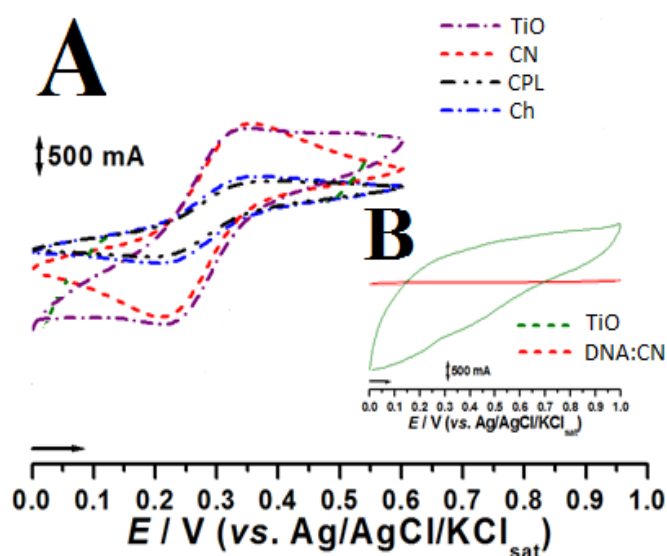

Figure 1. Cyclic voltammograms obtained for different modifiers CPLs in 0.05 M potassium ferrocyanide: 0.1 M PBS, pH 6.0 (A) and 0.1 M PBS, pH 6.0 (B). Scan range from 0 to 1.0 V, scan rate of 100 mV.s^{-1}.

Figure 1A shows voltammograms obtained in the 0.1 mM potassium ferricyanide in 0.1 M PBS, pH 6.0 at CPL biosensors. It was observed, that CN, TiO_2 and also DNA-CN (not shown) increase the electroactive surface area of CP electrodes, leading to high Faradayc peak currents. On the other hand, since inorganic clusters, such as TiO_2, as well as, most of the biopolymers possess lower conductive nature than "sp^2 like" carbon materials, the reversibility of Ferro/Ferricyanide redox probe may be compromised (Figure 1A) [25, 26].

In fact, the capacitive current observed in blank solution was lower for CPLs modified with activated CN (not shown) and DNA-CN, whereas the ascendant voltammetric profile observed for CPL-TiO reinforces that the metal oxide hampers the electrical conductance of electrode [24]. The same negative effect on the conductance was observed for the bioplolymers Ch and BSA. Meanwhile, as expected the decrease of added amount or removal of such modifiers improved the electrical properties. The relative DNA conductivity can be explained by their π-π type electrons and positively charged counter ions [27]. Nevertheless, even for DNA the amount and environmental conditions must be carefully optimized, otherwise it can be obtained opposite effects [27, 28].

3.2 Electrocatalytical Properties and Calibration Curves

The electrical properties of electrodic materials may influence the electrocatalytical properties against target analytes. Therefore, since laccase are polyphenoxidase enzymes, the different CPLs were evaluated against catechol. Thus, the biosensor performance was evaluated by means of cathodic peak current levels obtained for the biochemically generated quinone (Figure 2).
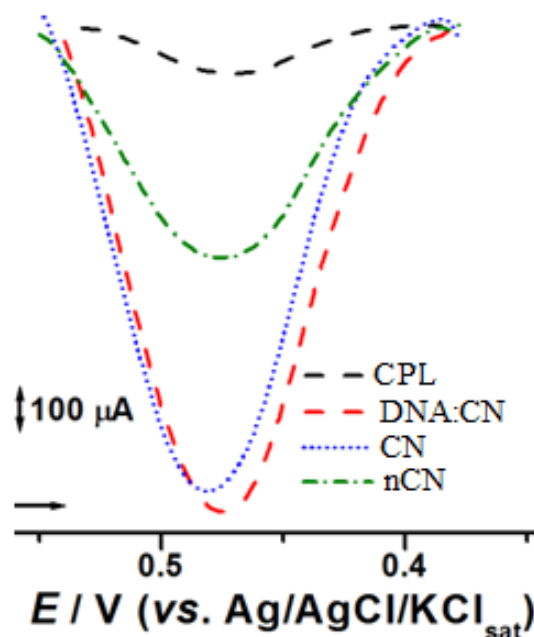

Figure 2. DP voltammograms obtained for different modified CPLs in 0.05 mM Catechol in 0.1 M PBS, pH 6.0. Pulse amplitude of 50 mV, scan rate of 10 mV.s^{-1}.

As expected, it can be observed from Figure 2, that activated CNs have great influence on the biosensor sensitivity. It is explained by the enhancement of electroactive surface area, increasing the electron transfer effectivity. In turn, the slight improvement achieved by DNA incorporation on the electrode, may be consequence of their good electrical conductance and great biocompatibility with enzyme, thus leading to better enzymatic activity at immobilized state. Moreover, Glu, Ch or BSA modifiers (not shown) do not have significant influence on the biosensor performance, whereas the non-activated CN exhibits far lower positive effect.

Table 2 enrolls the detailed merit data for each modified CPL evaluated, obtained from rutin calibration curves, herein represented in Figure 3 for CPL-DNA:CN.

The best biosensor performance expressed by low limits of detection (LOD) and quantification (LOQ), good coefficient of correlation, higher storage stability and lower peak potentials were obtained for CPL-DNA:CN biosensor (Figure 3).

Table 2. Merit parameters obtained from rutin calibration curves for different modified CPLs in 0.1 M PBS (pH 5.0).

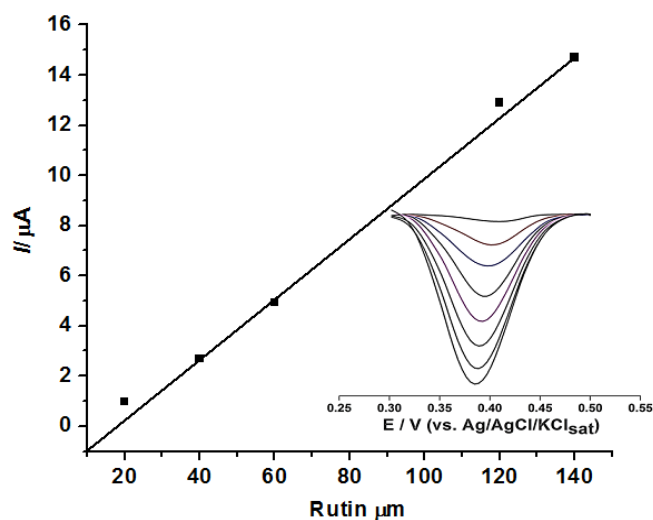| Carbon Paste Biosensors	E_{pc} (V)	R	LOD (µM)	LOQ (µM)	3 days (%)	10 days (%)
CPL	0.38	0.9373	30	91	69%	57%
CPL-Glut	0.38	0.9373	40	120	78%	69%
CPL-BSA	0.42	0.8521	29	89	73%	57%
CPL-Ch	0.38	0.9892	28	79	69%	63%
CPL-SiO	0.39	0.987	16	49	94%	83%
CPL-TiO	0.39	0.991	27	83	52%	31%
CPL-DNA	0.40	0.9896	19	69	92%	56%
CPL-nCN	0.39	0.9845	28	88	68%	58%
CPL-CN	0.39	0.9943	16	51	95%	94%
CPL-DNA:CN	0.39	0.9987	12	38	100%	100%

Figure 3. Calibration curve obtained from the DPVs, after successive additions of 1mM rutin standard solution at CPL-DNA:CN in 0.1 M Phosphate buffer (pH 5.0) solution. Scan range from 0.50 to 0.20 V; scan rate of 10 mV.s^{-1}; pulse amplitude of 50 mV.

The higher stability can be also explained by the bioaffinity between DNA and enzyme, whereas the higher surface area, as well as, the cylindrical shape of MWCNTs provides a suitable site for enzyme immobilization.

3.3 Optimization of Experimental Conditions and Affinity Assay

Since CPL-DNA:CN presented better performance, the influence of conditioning time and start potential, as well as the affinity assay were also evaluated.

Nevertheless, the higher the conditioning time, the higher the time for enzymatic oxidation will be, as well as the expected cathodic currents; the fastness of assay is also a desirable feature. In turn, the higher the start potential, the higher will be the chance to provoke electrochemical oxidation will be, hence the selectivity of a biosensor will be higher at lower values.

Therefore, since the conditioning time and starting potential dictate the rapidity and selectivity of analysis, but have opposite effect on the sensitivity, such parameters are very relevant and must be carefully adjusted.

Concerning to the selectivity, the laccase based biosensors have broad range of activity against phenolic compounds, but present higher kinetic rate against catechol like derivatives.

Figures 4A and 4B show the effect of conditioning time and start potential against catechol. In turn, Figure 4C presents the affinity assay against different phenolic compounds, at conditioning time of 30 s and starting potential of 0.5 V.
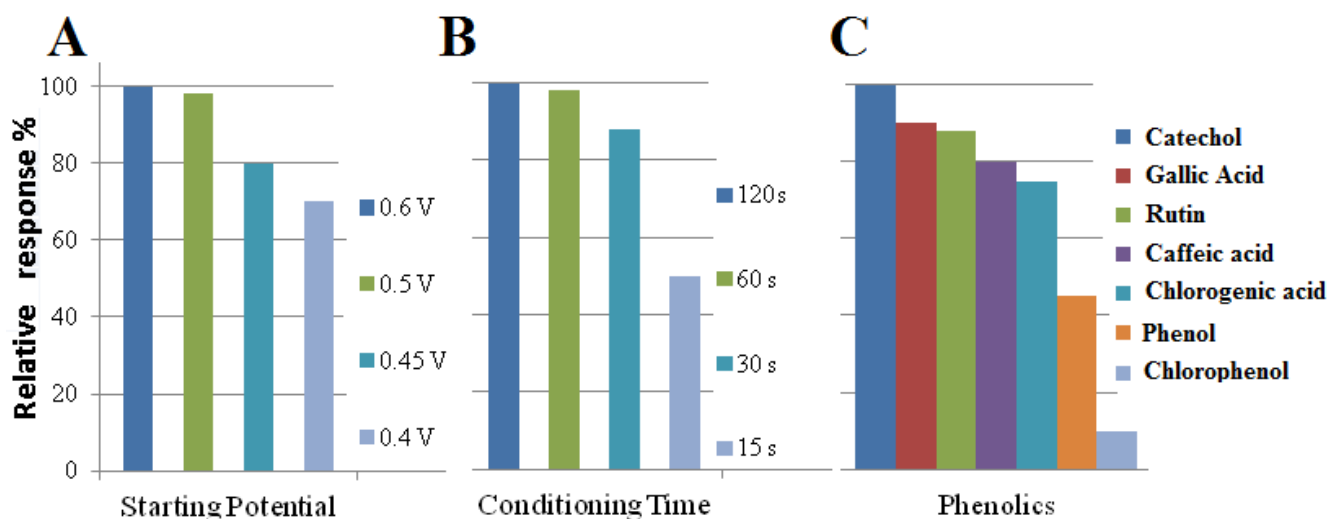

Figure 4. Effect of starting potential (A) and conditioning time (B) on the biosensor performance and the relative response of CPL-DNA:CN against equimolar concentrations of different phenolic compounds (C).

3.4 Application of Biosensor for determination of Antioxidant Capacity

The antioxidant capacity of many natural compounds is very close to the total phenolic (TP) content. Therefore, taking into account the good sensitivity of CPL-DNA:CN for many polyphenolic

antioxidants (Figure 4C), this biosensor was elected for analysis of total phenol (TP) content in coffee crude samples (Table 3).

Table 3. TP content values expressed as Gallic Acid Equivalents (GAEq) (mg/mL) obtained for different roasted coffee beverages by using CPL-DNA:CN biosensor and FC method.

Sample	CPL-DNA:CN GAEq (mg/mL)	FC GAEq (mg/mL)	DPPH (EC_{50})
SG (N = 5)	0.48 ± 0.1	0.54 ± 0.2	19.74 ± 1.94
SP (N = 5)	0.43 ± 0.1	0.53 ± 0.2	21.30 ± 0.52
PI (N = 5)	0.41 ± 0.1	0.42 ± 0.2	22.43 ± 1.07
E (N = 5)	0.53 ± 0.1	0.60 ± 0.3	19.72 ± 2.11

SG = soluble granulates; SP = soluble powder; PI = Powder for infusion and E = capsules for espresso.

The biosensor and FC results for TP values presented good agreement. Moreover, the RSD % obtained for the biosensor was lower than the one obtained for FC method. Finally, as expected for coffee samples the TP content agreed with EC_{50} values obtained for DPPH assay.

Furthermore, the performance of CPL-DNA:CN was similar to other laccase based biosensor, in which despite to the higher complexity concerning their preparations, the limit of detection for phenolic antioxidants have also been in the μM level [29,30].

4. CONCLUSIONS

The biopolymers, specially the DNA confers higher stability to laccase biosensors, whereas MWCNTs increased the electroactive area and improved the electrical properties leading to higher Faradaic peak currents. The optimized CPL-DNA:CN biosensor showed good performance on the determination of natural phenolics, being a useful and easy tool for TP and antioxidant capacity determinations.

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