Food Chemistry 315 (2020) 126306

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Chemometric-assisted construction of a biosensing device to measure chlorogenic acid content in brewed coffee beverages to discriminate quality

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ARTICLE INFO

Keywords:
Statistical mixture design
Biosensor architecture
Bothyosphaeria rhodina MAMB-05
Platinum nanoparticles
Voltammetric fingerprint
Coffee beverages

ABSTRACT

In this work we propose the use of statistical mixture design in the construction of a biosensor device based on graphite oxide, platinum nanoparticles and biomaterials obtained from *Botryosphaeria rhodina* MAMB-05. The biosensor was characterized by electrochemical impedance spectroscopy. Under optimized experimental parameters by factorial design, the biosensor was applied to the voltammetric determination of chlorogenic acid (CGA) measured as 5-O-caffeoylquinic acid (5-CQA). The biosensor response was linear (R^2 = 0.998) for 5-CQA in the concentration range 0.56–7.3 μmol L^{-1}, with limit of detection and quantification of 0.18 and 0.59 μmol L^{-1}, respectively. The new biosensing device was applied to quality control analysis based upon the determination of CGA content in specialty and traditional coffee beverages. The results indicated that specialty coffee had a significantly higher content of CGA. Principal component analysis of the voltammetric fingerprint of brewed coffees revealed that the laccase-based biosensor can be used for their discrimination.

1. Introduction

Coffee is one of the most consumed beverages worldwide. Its consumption is correlated with several health benefits linked to the reduced risk of Alzheimer's, Parkinson's, type 2 diabetes mellitus and cardiovascular diseases (Gökcen & Şanlier, 2019). Its health benefits depend on chlorogenic acids (CGA), caffeine, caffeic acid, among other compounds (Gökcen & Şanlier, 2019). These compounds as well as carbohydrates, lipids and vitamins present are directly related to the quality of coffee. The ratio of each compound is dependent upon several factors that can include the type of coffee cultivar (*Coffea* spp.), the harvesting method, and the roasting procedures of the raw coffee beans. The brewing process may also have influence (Angeloni et al., 2019; Cheng, Furtado, Smyth, & Henry, 2016; Guizellini et al., 2018).

Among more than 950 compounds present in roasted coffee beans (Farah, 2012), the CGA feature prominently. They are polyphenolic esters of caffeic, ferulic and quinic acids that give rise to a family of *O*-caffeoylquinic (CQA), *O*-feruloylquinic and *O*-dicaffeoylquinic acids that exist in three isomeric forms at the 3, 4, and 5 positions on the quinic acid moiety (Bicho, Lidon, Ramalho, & Leitão, 2013). These polyphenolic compounds present several healthful properties among which are lowering of blood pressure, improve regulation of blood glucose levels, protect against cardiovascular disease and atherosclerosis, and display anti-inflammatory and antioxidant activity (Jeon et al., 2019), and their concentration is related to the quality of the coffee (Clifford, 1997; Farah, Monteiro, Calado, Franca, & Trugo, 2006; Leroy et al., 2006).

Their content in brewed coffee beverages has been determined by several analytical methods that include spectrophotometry (Moores, McDermott, & Wood, 1948) and high-performance liquid chromatography (HPLC) (Angeloni et al., 2019; Jeon et al., 2017, 2019; Zanin, Corso, Kitzberger, Scholz, & Benassi, 2016). The number of publications regarding the electroanalytical determination of CGA have increased in the last years, mainly through the use of biosensors (Amatatongchai, Sroysee, Laosing, & Chairam, 2013; Fernandes et al., 2009; Litescu, Eremia, Bertoli, Pistelli, & Radu, 2010; Rodríguez-Delgado et al., 2015). The employment of biosensors is important in the electrochemical analysis of complex matrices such as coffee, since they allow the

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https://doi.org/10.1016/j.foodchem.2020.126306
Received 19 September 2019; Received in revised form 24 January 2020; Accepted 26 January 2020
Available online 28 January 2020

selective and rapid determination of the target analyte at reduced instrumentation and analytical costs.

In this context, laccase (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase), a multicopper enzyme catalyzing the oxidation of phenolic substrates in the reduced form, has been employed in biosensors to measure phenolic compounds (Rodríguez-Delgado et al., 2015). Laccases are ubiquitously produced by different microbial and plant species, and is easily obtained from the fungus *Botryosphaeria rhodina* MAMB-05 cultured on basal nutrient medium with veratryl alcohol as enzyme inducer (Vasconcelos, Barbosa, Dekker, Scarminio, & Rezende, 2000). This enzyme in crude form has previously been employed in biosensors for the determination of the catecholamines, epinephrine (Moraes et al., 2019), dopamine (Coelho et al., 2019), and hydroquinone (Mattos et al., 2019). The laccase biosensor catalyzed the oxidation of the catecholamines to the correspondent *o*-quinones, which were then reduced by applying a potential sweep to the electrode. For this purpose, the enzyme is immobilized onto a support on the surface of an electrode. It is known that enzyme immobilization under conditions close to those of its natural environment guarantee a more stable and active enzyme, and is an important property when considering its use in biosensors.

In previous reports on electrochemical sensoring devices from our research group, we focused on using botryosphaeran (BOT) as a platform material (Coelho et al., 2019; Eisele et al., 2019; Mattos et al., 2019). BOT is an exopolysaccharide of the (1 → 3)(1 → 6)-β-D-glucan type produced by *B. rhodina* MAMB-05 (Dekker, Queiroz, Cunha, & Barbosa-Dekker, 2019). Nanomaterials are considered promising transduction candidates attributable to their electrochemical properties: fast electron transfer, increased sensitivity and lower limits of detection of the analytes (Holzinger, Le Goff, & Cosnier, 2014; Mattos et al., 2019).

The ratio of materials used in the construction of biosensing devices are usually randomly determined by univariate experiments. Univariate experiments (a “one-factor-at-a-time” approach) do not deal with interactions between materials, and the electrochemical response is not optimally covered by the chosen experimental parameters. An optimal coverage of the electrochemical response region is guaranteed when simultaneously varying all of the materials that influence the response of the constructed biosensor, This coverage is possible through the Design of Experiments (DOE), where a minimum number of experiments produce the maximum amount of information (Scarminio, Barros, & Bruns, 2001; Marcheafave et al., 2019). Thus, considering that the use of factorial experiments and statistical mixture design have not been exploited for this purpose, the objective of the work presented herein was to use the simplex-centroid design for the construction of a biosensor, and use the DOE in the development of the voltammetric method. The fabricated biosensor was applied in the determination and comparison of CGA in specialty and traditional roasted coffees to discriminate for quality control purposes.

2. Materials and methods

2.1. Chemicals and solutions

All chemicals used in this work were of analytical grade and used as received, and all solutions were prepared using ultra-purified water (resistivity ≥18.2 MΩ cm) obtained from a Milli-Q system (Millipore, USA).

5-*O*-caffeoylquinic acid (5-CQA, employed as a CGA standard), caffeic acid, ferulic acid, *p*-coumaric acid, ascorbic acid, chloroplatinic acid (H_2PtCl_6), polyvinylpyrrolidone (PVP, MW 55,000 g mol^{-1}), dimethylformamide (DMF), and graphite powder (< 0.1 mm) were obtained from Sigma-Aldrich (St Louis, MO, USA). The supporting electrolyte for voltammetric measurements comprised a 0.10 mol L^{-1} phosphate buffer solution with pH adjusted to 4.0 by mixing 0.1 mol L^{-1} in KH_2PO_4 and K_2HPO_4, both obtained from Labsynth (Diadema, SP, Brazil). 5-CQA stock solutions of 10 mmol L^{-1} were freshly prepared by dissolving the standard in ultra-pure water. Working solutions were prepared by diluting the stock solution with the supporting electrolyte.

2.2. Instrumentation

All voltammetric measurements were conducted using a PGSTAT 101 potentiostat/galvanostat (Metrohm Autolab B. V., Schiedam, Netherlands) controlled by NOVA 2.1 software. A conventional three-electrode glass cell was employed using a platinum plate as the auxiliary electrode, Ag/AgCl (3.0 mol L^{-1} KCl) as the reference electrode, and the constructed biosensor as the working electrode.

The electrochemical impedance spectroscopy (EIS) experiments were carried out using a FRA II μAutolab type III potentiostat/galvanostat (Metrohm Autolab B. V., Schiedam, Netherlands) controlled by NOVA 1.10 software. The experiments were performed at the formal potential of the $[Fe(CN)_6]^{3-/4-}$ redox pair (0.322 V for the electrodes employed in this work), from 10 mHz to 100 KHz (10 points per decade), and with a 10 mV (rms) ac perturbation. The solution was composed of 0.005 mol L^{-1} $K_3[Fe(CN)_6]$, 0.005 mol L^{-1} $K_4[Fe(CN)_6]$ and 0.5 mol L^{-1} KCl.

The pH of solutions was measured using a pH meter (model W3B; BEL Engineering, Milan, Italy) employing a combined glass electrode with an Ag/AgCl (3.0 mol L^{-1} KCl) external reference electrode.

HPLC analyses were carried out using a Finnigan Surveyor 61,607 HPLC system coupled with a Plus photodiode array detector (PDA) (Thermo Fisher Scientific Inc., San Jose, USA). Chromatographic conditions used in this study were as outlined by Vinson, Chen, and Garver (2019) with some modifications. Chromatographic separation was achieved on an ACE 5 C-18 column (250 mm × 4.6 mm i.d., particle size: 5 μm) at 24.0 (± 0.1) °C. The 5-CQA standard was used for the calibration curve. The detector was set at 325 nm and the retention time of 5-CQA was of 18 min. 5-CQA was identified by the time of elution, and the ultraviolet (UV)-visible spectrum by PDA detection.

2.3. Preparation of graphite oxide paste

Graphite oxide was prepared by acid treatment at room temperature according to Wong et al. (Wong, Silva, & Fatibello-Filho, 2017). For this purpose, 1.0 g d.w. of graphite powder was added to 200 mL of a mixture of H_2SO_4 and HNO_3 (ratio 1:1, v/v), and the mixture stirred for 12 h. Thereafter, with the aid of a centrifuge, the graphite oxide preparation was extensively washed with ultrapure water until the pH reached 6.5 followed next by drying at 90 °C. A paste of graphite oxide was prepared by mixing graphite oxide and mineral oil (Nujol®) in a ratio of 70:30% (w/w).

2.4. Production of laccase and botryosphaeran by Botryosphaeria rhodina MAMB-05

Laccase and botryosphaeran (BOT) were obtained from the ascomyceteous fungus, *Botryosphaeria rhodina* MAMB-05. Laccase was produced under conditions of submerged fermentation on basal medium containing the inducer veratryl alcohol (30.4 mmol L^{-1}) as outlined by Vasconcelos et al. (2000), while BOT was produced on medium containing 6% sucrose as described by Steluti et al. (2004). BOT was isolated from the cell-free fermentation broth by precipitation with ethanol, and the recovered precipitate redissolved in water while stirring for 2 h at 60 °C. Laccase and BOT recovered were exhaustively dialyzed against several changes of distilled water over 48 h, followed by lyophilization of both preparations. The crude lyophilized laccase preparation (LCE) was dissolved in ultra-purified water at a concentration of 1.0 mg mL^{-1} (0.33 units) and kept frozen (-20 °C), while

an aqueous solution of BOT (0.113 g L^{-1}) was stored under refrigeration (~4 °C) until required.

2.5. Synthesis of platinum nanoparticles

In order to synthesize nanoparticles, 12 mg of ascorbic acid and 70 mg of PVP were dissolved in 12 mL of water, and after complete dissolution, the mixture was stirred at 90 °C for 10 min. Thereafter, 6 mL of a 3.0 mmol L^{-1} H_2PtCl_6 solution was rapidly added to the mixture producing a black suspension after 10 min. The reaction was continued for 30 min, and then was followed by a further addition of 6 mL of H_2PtCl_6 (3.0 mmol L^{-1}), and after leaving the reaction mixture for a further 30 min, the nanoparticles were ready (Fig. S1, supplementary material). The nanoparticles were then washed with acetone (3 times) and water (twice), and aided by centrifugation, the PtNPs were recovered. For use, the prepared PtNPs were resuspended in water/DMF (ratio 1:1, v:v) using ultrasound. The platinum content in the suspension was 0.16 mg mL^{-1}, quantified using flame atomic absorption spectroscopy.

2.6. Biosensor construction

The prepared graphite oxide paste was carefully packed into the cavity (diameter of 3.0 mm) of a Teflon® tube. The graphite oxide paste electrode was then positioned vertically with the paste surface turned upwards to allow the dropping of the biosensor components (PtNPs, laccase and BOT) onto the electrode surface (see below). The graphite oxide paste electrode presented an exposed geometric area of 0.0707 cm^2.

The optimum architecture for constructing the biosensor was determined by statistical mixture design (see below), where the optimum ratio was 1:6:1 for PtNPs, BOT and laccase, respectively. The biosensor was fabricated in two steps: firstly, 3 μL of the PtNPs suspension was deposited with the aid of a micropipette onto the surface of the prepared graphite oxide paste electrode and left to dry at room temperature for 1 h. Afterwards, 12 μL of BOT solution followed by 3 μL of laccase were sequentially dropped onto the surface containing the layered PtNPs. The biosensor was then left to dry at ~4.0 °C. Other sensors were fabricated for characterization and comparative purposes using the design matrix as shown in Table S1.

2.7. Design of experiments and statistics

Conditions to optimize the parameters to construct a biosensor to measure CGA content were performed using a statistical mixture design matrix (Scarminio et al., 2001) with three components (BOT, PtNPs and laccase) in the formulation with 10 experimental runs (Table S1 – supplementary material). In a mixture experiment, the sum of the component fractions must be equal to unity and their proportions must be non-negative. The restrictions on the levels of each factor are expressed as follows (Eq. (1)):

$$\sum_{i=1}^{q} x_i = x_{BOT} + x_{PtNPs} + x_{LCE} = 1 \; or \; 100\% \quad (1)$$

where x_{BOT}, x_{PtNPs} and x_{LCE} represents the proportion of the components in the mixture, and $q = 3$ is the number of components (Scarminio et al., 2001). The advantage of using this systematic approach in the construction of a biosensor device lies on the modeling of data through typical canonical models to represent the voltammetric response of the biosensor as a function of the components and predicting the interactions between them. The model that describes the largest number of interactions between these components is the full cubic model.

The full cubic model for a mixture of three components is given by the simplified generic expression (Eq. (2)),

$$\hat{y} = b_1^* x_1 + b_2^* x_2 + b_3^* x_3 + b_{12}^* x_1 x_2 + b_{13}^* x_1 x_2 + b_{23}^* x_2 x_3 + d_{12}^* x_1 x_2 (x_1 - x_2) + d_{13}^* x_1 x_3 (x_1 - x_3) + d_{23}^* x_2 x_3 (x_2 - x_3) + b_{123}^* x_1 x_2 x_3 \quad (2)$$

where $\hat{y}$ is the predicted response as a function of the components used, x_1, x_2 and x_3, and their respective regression coefficients (b*and d*). The first three terms of the expression describe the contribution of pure components (b_1^*, b_2^*, b_3^*), the other terms describe the contribution resulted from the interaction between pairs of components (b_{12}^*, b_{13}^*, b_{23}^*), and the last term describes the contribution resulting from cubic coefficient of the binary synergism (d_{12}^*, d_{13}^*, d_{23}^*) and the interaction between three components on the final response (b_{123}^*). In the case of the biosensor, a positive term in this equation indicates antagonistic interactions, while negative terms indicate synergistic interactions in the final biosensor response. The quality of the mixture models are judged by Analysis of Variance (ANOVA) of the regression data.

The independent variables for optimization of parameters in designing an electrochemical sensoring device that measures 5-CQA were: BOT (x_1), PtNPs (x_2) and LCE (x_3) as described in Table S1 (supplementary material).

The optimization of the experimental and operational parameters for the voltammetric determination of CGA by the biosensor consisted on a 2^4 factorial design with central point (evaluating pH, amplitude (a), frequency (f) and potential step (ΔE) with 17 experimental runs) followed by a central composite design experiment (evaluating a, f and ΔE with 15 experimental runs). The optimum conditions were chosen based upon the higher response of the biosensor for the electroreduction of 5-CQA (see Table 1, Section 3.3).

ANOVA and multiple regression analyses were performed using Statistica 7.0 software (http://www.statsoft.com). Statistical analyses were performed using Origin® software (https://www.originlab.com) at the 95% confidence level. Principal component analysis was performed using MATLAB software version R2007b (www.mathworks.com) and PLS Toolbox software version 5.8.1 (http://eigenvector.com/software/pls-toolbox/) for pre-processing, analysis and, visualization.

2.8. Coffee samples and their preparation

The fabricated biosensor was applied to determine CGA in brewed coffees. Coffee samples (*Coffea arabica*) were classified as traditional or specialty types. The traditional coffees were of different brands purchased from a local supermarket. The specialty coffees (Agrton roasting score 55, see: https://www.coffeeenterprises.com/) were classified as very good quality accordingly to the protocols of the Specialty Coffee Association of America (SCAA) with a score of 83.3 ± 0.9, and were obtained from the International Fair of Specialty Coffees (March 2016), in Jacarezinho-PR, Brazil.

Brewed coffee beverages were prepared as follows: 10 g of each grounded coffee sample was transferred into a coffee paper filter. To this was added 100 mL of hot tap water (97 °C) by carefully pouring the water onto the coffee powder ensuring to wet all the sample contained in the filter, and the filtrate collected represents the brewed coffee sample.

For electrochemical analysis, an aliquot of 25 uL of the brewed coffee was directly transferred to the electrochemical cell. For analysis by HPLC, the brewed coffee was first filtered through a PTFE-20/25 0.20 μm membrane (Chromafil® Xtra), and then directly injected into the chromatographic system.

3. Results and discussion

The production of laccase and botryosphaeran (BOT) is dependent upon the nutrient medium on which the fungus *B. rhodina* MAMB-05 is grown. When cultured on basal medium (glucose and minimum salts) in the presence of the laccase inducer, veratryl alcohol, laccase is produced above constitutive levels (Dekker & Barbosa, 2001), but under these conditions, the presence of inducer interfered with the synthesis

Table 1
Experimental matrix and results for the optimization of pH and operational parameters for measuring 5-CQA by the biosensor.

Factors	Factorial design (2^4)					Central Composite design (2^3)				
	Levels					Levels				
	Inferior (−)	Superior (+)		Central (0)		Inferior (−)	Superior (+)		Central (0)	
pH	4	8		6		*a* (mV)	80	120	100	
a (mV)	25	100		62.5		*f* (s^{-1})	20	40	30	
f (s^{-1})	30	50		40		Δ*E* (mV)	5	9	7	
Δ*E* (mV)	3	7		5						
Experiment	Coded levels				Response (−μA)	Experiment	Coded levels			Response (−μA)
	pH	*a*	*f*	Δ*E*			*a*	*f*	Δ*E*	
1	−	−	−	−	17.4 ± 0.50	1	−	−	−	91.5 ± 0.60
2	+	−	−	−	5.49 ± 0.25	2	+	−	−	115 ± 0.54
3	−	+	−	−	58.9 ± 0.71	3	−	+	−	83.2 ± 0.03
4	+	+	−	−	14.3 ± 0.61	4	+	+	−	113 ± 0.09
5	−	−	+	−	15.1 ± 0.31	5	−	−	+	106 ± 0.82
6	+	−	+	−	5.81 ± 0.20	6	+	−	+	126 ± 0.22
7	−	+	+	−	54.2 ± 0.62	7	−	+	+	87.1 ± 0.91
8	+	+	+	−	15.6 ± 0.24	8	+	+	+	120 ± 0.54
9	−	−	−	+	29.1 ± 0.15	9	1.68	0	0	112 ± 0.87
10	+	−	−	+	8.35 ± 0.10	10	−1.68	0	0	87.2 ± 0.62
11	−	+	−	+	**127 ± 0.27**	11	0	1.68	0	103 ± 0.45
12	+	+	−	+	23.4 ± 0.18	12	0	−1.68	0	126 ± 1.1
13	−	−	+	+	23.3 ± 0.07	13	0	0	1.68	118 ± 0.71
14	+	−	+	+	6.42 ± 0.12	14	0	0	−1.68	72.8 ± 0.59
15	−	+	+	+	75.6 ± 0.41	**15**	**0**	**0**	**0**	**127 ± 0.27**
16	+	+	+	+	20.3 ± 0.37					
17	0	0	0	0	34.6 ± 0.25					

of BOT resulting in diminished amounts (Dekker, Vasconcelos, Barbosa, Giese, & Paccola-Meirelles, 2001). When cultured on sucrose medium, BOT levels increased substantially (Steluti et al., 2004), but this was also accompanied by low levels of laccase (Cunha, Barbosa, Giese, & Dekker, 2003). Thus, taking into account the production of either bioproduct, laccase and BOT coexist in the fermentation broth, which represents a natural habitat for enzyme and the exopolysaccharide. BOT presents film properties which can serve in immobilizing laccase in biosensors (Coelho et al., 2019; Mattos et al., 2019).

Considering that PtNPs present interesting electrocatalytic features (Ramírez-Meneses et al., 2009; Song, Zhen, Yin, & Song, 2019), our intention was to fabricate a biosensor device using a statistical mixture-design matrix in which layers of PtNPs, BOT and laccase were sequentially deposited on top of a graphite oxide paste electrode, and to employ the developed biosensor to electrochemically determine the CGA content in coffees; an indication of coffee quality (Farah et al., 2006; Leroy et al., 2006; Zanin et al., 2016).

The developed biosensor device was calibrated against 5-CQA, as it is the major constituent of CGA group in coffee, and therefore representative of coffee quality.

3.1. Statistical mixture design for the construction of the biosensor

The construction of the biosensor architecture was examined using three components (BOT, PtNPs and laccase) arranged in a statistical mixture design (Table S1 – supplementary material). A total of ten sensoring devices were constructed with different ratios of the components (Fig. 1A). In constructing the biosensor, three architectures corresponding to pure components (vertex points – white circles), three to a binary mixture of two components (edge points – black circles), three to the ternary mixture with different ratios (axial points – white rhombus), and the last corresponding to the ternary mixture (center point – black rhombus), which was run in triplicate for evaluation of statistical confidence. The responses of the biosensor were evaluated in terms of the square-wave (*a*: 40 mV; *f*: 30 s^{-1}; Δ*E*: 2 mV) voltammetric current obtained with 99.0 μmol L^{-1} of 5-CQA in phosphate buffer solution (pH 6.0). The current response of the biosensor based upon the mixture design was analyzed by increasing complexity of statistical models, starting with the linear model, then the quadratic, followed by the special cubic, and full cubic models based upon the analysis of variance at the 95% confidence level. Table S2 (supplementary material) presents the ANOVA results for all of the models considered. The full cubic model was observed to present suitable significance of regression and slight lack of fit. Fig. 1B presents the response surface plot with the simplified cubic model (Eq. (3)) of the experimental response that indicates the current obtained in the experiments, where the lower the current presented the higher response of the biosensor.

$$\begin{aligned}\hat{y}_{current} &= \underset{(\pm 0.60)}{-14.70BOT} - \underset{(\pm 0.60)}{6.31LCE} - \underset{(\pm 0.60)}{19.15PtNPs} + \underset{(\pm 2.93)}{6.78BOT.LCE} \\ &+ \underset{(\pm 2.93)}{12.36LCE.PtNPs} - \underset{(\pm 9.73)}{82.10BOT.LCE(BOT-LCE)} \\ &- \underset{(\pm 9.73)}{182.81BOT.PtNPs(BOT-PtNPs)} - \underset{(\pm 19.22)}{75.21BOT.LCE.PtNPs}\end{aligned} \quad (3)$$

Equation (1) defines the biosensor predicted current response depending upon the proportion of the BOT, PtNPs and LCE components ($\hat{y}_{current}$). According to Eq. (1), the most important linear coefficients were PtNPs and BOT followed by LCE. The binary combinations (BOT-LCE, LCE-PtNPs) showed an antagonistic effect on the biosensor response, but the ternary interaction (BOT-LCE-PtNPs) had a synergic effect on the response, in other words, when the three materials are associated, larger responses were achievable. Cubic coefficients of binary synergism showed a major significant effect for BOT.PtNPs(BOT-PtNPs), than that observed for BOT.LCE(BOT-LCE). Ternary interaction is shown in the curvature of the response surface proximate to experimental run number 7 of the mixture design (Fig. 1B and Table S2). The presence of PtNPs contributes to a higher response since it presents electrocatalytic features and higher conductivity (Ramírez-Meneses et al., 2009; Song et al., 2019). Associated with this feature, the combination between BOT and LCE indicates that a small quantity of the enzyme placed in its natural environment (i.e., with BOT) results in a

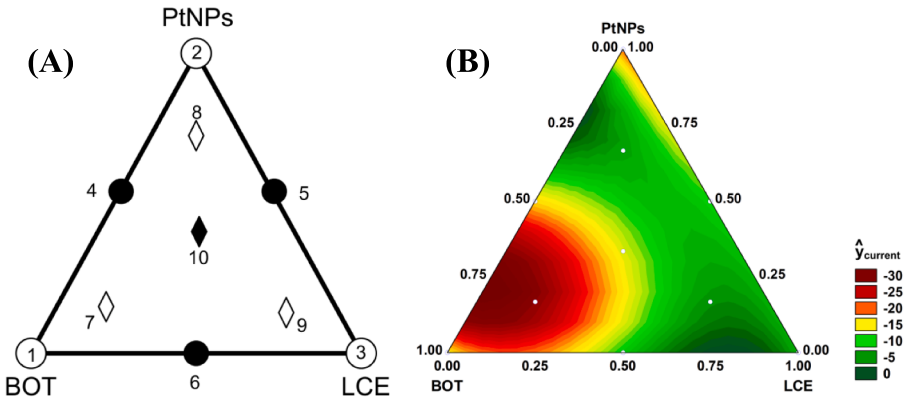

Fig. 1. (A) Statistical mixture design using pure (○), binary (●) and ternary (◊ – different ratio and, ◆ – equal ratio) components for construction of the biosensor. The experimental runs used are numerically indicated. (B) Response surface plot for construction of the biosensor using PtNPs, BOT and laccase (LCE). The colors indicate the predicted current obtained on reduction of 5-CQA by the biosensor.

higher responsive biosensor.

The cubic regression model (Eq. (3)) it showed slight lack of fit at the 95% confidence level ($MQ_{lof}/MQ_{ep} = 4.90 > F_{1,20} = 4.35$), according to the ANOVA results. This observation indicates that model can be used to predict response values for the experimental domain. However the confidence intervals for predictions could be slightly broader than indicated by the 95% level. The model also has R^2_{adj} higher than 98%. According to this, and considering the highest experimental response obtained in the axial point 7 (2/3 BOT; 1/6 PtNPs and 1/6 laccase (LCE); Table S1; Fig. 1B), the combined ratio of the three components was employed for constructing the biosensor.

3.2. Spectroscopy characterization of the biosensor

The properties of each component added in building the biosensor were evaluated by EIS measurements. The EIS technique is based upon the measurement of impedance (ratio between voltage and current) varying with the frequency of a perturbation. Its results, expressed on the Nyquist Plot can lead to interpreting the biosensor surface in terms of an electrical circuit with the combination of the circuit elements resistor, capacitor and/or inductor. The diameter of the semicircle formed in the region of high frequency of the Nyquist diagram is attributed to the electron transfer resistance (R_{ct}) value of the analyzed interface (Orazem & Tribollet, 2008). Spectra were obtained for the bare graphite oxide paste electrode before and after the sequential addition of the biosensor components, in 0.01 mol L^{-1} solution of $[Fe(CN)_6]^{3-/4-}$, and the frequency range of 100 kHz to 0.1 Hz, under the application of 0.322 V vs Ag/AgCl (Fig. 2). The addition of PtNPs to the graphite oxide paste electrode was observed to cause a decrease in the R_{ct} from 56.2 to 44.7 Ω. This is related to the high conductivity and electrocatalytic properties of the metallic nanoparticles (Holzinger et al., 2014). The addition of BOT and LCE was responsible for an increase in the R_{ct} to 63.8 and 67.2 Ω, respectively. The results obtained are in agreement with previous observations on the poor conductivity properties of BOT and laccase (Coelho et al., 2019; Eisele et al., 2019; Mattos et al., 2019).

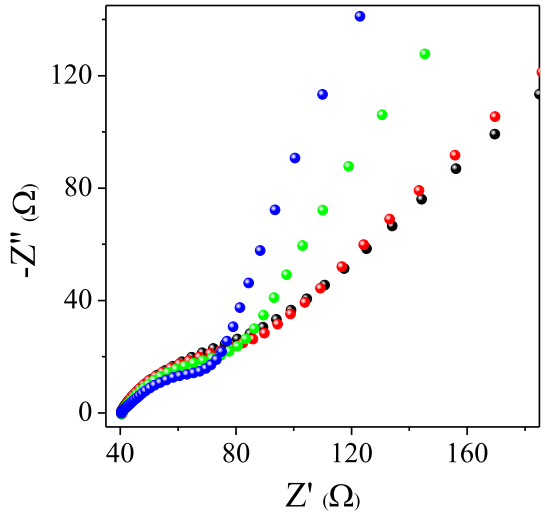

Fig. 2. Nyquist diagram of graphite oxide paste electrode (green) and its modification with PtNPs (blue), PtNPs and BOT (red), and PTNPs, BOT and LCE (black), in 0.01 mol L^{-1} $[Fe(CN)_6]^{3-/4-}$ in 0.5 mol L^{-1} KCl solution in the frequency range from 100 KHz to 0.1 Hz. $E_{applied}$ = 0.32 V vs. Ag/AgCl. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

When comparing the biosensor composed of three components and the sensor composed of only PtNPs and BOT (according to experimental run number 7 without laccase addition, Table S1, supplementary material), we found that the biosensor presented a higher R_{ct} value since it contained a larger quantity of biomolecules with insulating characteristics. However, the response of the biosensor for the voltammetric reduction of 99.0 μmol L^{-1} 5-CQA in phosphate buffer solution (pH 4.0) was higher than the sensor with two components ($I_{biosensor} = -127 \pm 0.27$ μA and $I_{sensor} = -106 \pm 1.4$ μA). This observation highlights and confirms the role of laccase in the catalytic oxidation of polyphenols (Moraes et al., 2019; Rodríguez-Delgado et al., 2015). It is important to mention that our data were obtained after the optimization of the operational parameters of SWV, which is described in the next section.

3.3. Factorial design for the optimization of the chlorogenic acid response

Depending upon the pH, the laccase activity as well as the voltammetric response for the reduction of 5-CQA is different, and consequently the effect of pH was evaluated. As the voltammetric response of the biosensor is directly related to the operational parameters of the SWV, a full 2^4 factorial design with central point (Table 1) followed by another central composite design experiment (Table 1) was employed. The method optimization consisted of several experiments varying the pH and the operational parameters of SWV (amplitude (a), frequency (f) and potential increment, ΔE) at two different levels with a central point, for the factorial design. For the central composite design, the operational parameters of SWV were studied in a more specified range. Table 1 presents the studied parameters and the data obtained for each experiment. As can be seen, the lower the pH and the f values produced the higher voltammetric responses. On the otherhand, the higher the a and ΔE values, the higher the voltammetric responses. Therefore, the pH was fixed at 4.0, and the operational parameters (a of 100 mV, f of 30 s^{-1} and ΔE of 7 mV) became the center point of the central composite matrix design, with the parameters varied according to Table 1. High values of a and ΔE and low values of f increased the biosensor response, but at the same time, higher values of a and ΔE were

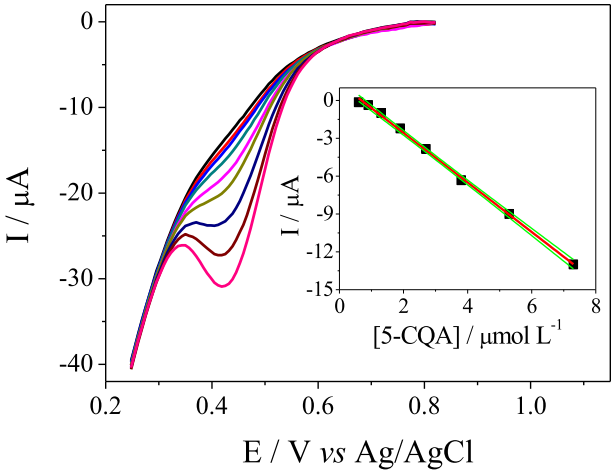

Fig. 3. Square-wave voltammograms obtained by the laccase biosensor for different concentrations of 5-CQA (0.56–7.3 μmol L^{-1}) in phosphate buffer solution (pH 4.0). *Inset*: the respective analytical curve with linear fitting (red line) and the confidence bands for 95% confidence level (green lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

responsible for an increase in the background current, causing deformation of the peak shape. These effects were statistically confirmed for the 2^4 factorial and 2^3 central composite designs as shown in Table S3 and S4 (supplementary material). For both the factorial and the central composite designs, all effects were significant, except for the amplitude × potential increment interaction for the central composite design. Consequently, the conditions of experiment 15 (pH = 4.0, a = 100 mV, f = 30 s^{-1} and ΔE = 7 mV) were chosen for the measurement of 5-CQA, as a higher response was observed with no peak shape deformation.

3.4. Analytical curve for 5-CQA using the biosensor, and evaluation of the biosensor lifetime and storage stability

With the use of the best operational parameters (experimental run n° 15, 2^3 Central Composite design, see Table 1), the analytical curve based upon the voltammetric response of the biosensor for 5-CQA was achieved in phosphate buffer solution at pH 4.0. Under these conditions, square-wave voltammograms were obtained (in triplicate) after the addition of small aliquots of the 5-CQA working solution into the electrochemical cell containing 10 mL of the supporting electrolyte. The voltammograms and the analytical curve are presented in Fig. 3.

The biosensor response was linear (R^2 = 0.998) within the 5-CQA concentration range of 0.56–7.3 μmol L^{-1}. The corresponding equation was I = 1.36 μA − 1.96 [5-CQA]/(μmol L^{-1}). The limit of detection and quantification were calculated based on the signal-to-noise ratio and the values obtained were 0.18 and 0.59 μmol L^{-1}, respectively.

The lifetime and storage stability of the biosensor was evaluated by measuring the square-wave voltammetric response of 1.99 μmol L^{-1} 5-CQA using the laccase-based biosensor for 30 days (five measurements per day), without any superficial renovation. When not in use, the biosensor device was kept under refrigeration (~4 °C) until the next analysis. The biosensor response decreased 3.48% at the end of the 7th day (35 measurements) and 6.59% at the end of the 30th day (150 measurements). The results indicated that the developed laccase-based biosensor has excellent storage stability, and a long lifetime when comparing to other described laccase-based biosensors (Arslan, Durmuş, Çolak, & Arslan, 2015; Huang, Fang, Liu, Gu, & Jiang, 2008; Moraes et al., 2019).

Table 2
Results obtained from the analyses of the 5-CQA content in brewed traditional and specialty coffees by the voltammetric biosensor and compared with the HPLC method.

Coffee sample[a]	5-CQA content[b,c]		Relative error (%)[d]	$F_{calculated}$[e]
	HPLC	Biosensor/SWV		
S1	330.2 ± 9.4	326.2 ± 9.3	−1.19	1.02
S2	360.5 ± 7.6	377.8 ± 3.3	4.78	5.39
S3	402.6 ± 9.1	401.3 ± 5.3	−0.31	2.99
S4	236.1 ± 8.6	223.9 ± 9.3	−5.14	1.17
S5	380.3 ± 9.3	361.9 ± 6.5	−4.84	2.76
S6	361.5 ± 6.5	366.3 ± 3.9	1.32	2.75
S7	322.5 ± 1.9	318.1 ± 4.1	−1.36	4.71
T1	264.3 ± 7.3	271.5 ± 4.5	2.70	2.63
T2	258.4 ± 6.4	259.6 ± 5.4	0.47	1.40
T3	227.4 ± 8.2	212.8 ± 2.8	−6.42	8.55
T4	298.2 ± 3.3	312.3 ± 7.8	4.73	5.81
T5	239.0 ± 2.1	233.0 ± 1.3	−2.49	2.55
T6	324.3 ± 4.6	317.4 ± 4.9	−2.12	1.15
T7	261.1 ± 6.2	267.2 ± 8.7	2.32	1.98

[a] S: specialty coffee; T: traditional coffee.
[b] Concentration is given in mg per 100 mL of brewed coffee.
[c] Performed in triplicate.
[d] Relative Error = 100% × (biosensor – HPLC)/HPLC.
[e] $F_{theoretical}$ = 19.0.

3.5. Analysis of specialty and traditional coffee beverages

The voltammetric laccase biosensor was applied to the determination of CGA in brewed specialty and traditional coffees. Beforehand, an interference study was conducted in order to identify possible interference by other components in the coffee samples during the electrochemical analyses by SWV. Compounds such as ferulic acid, *p*-coumaric acid, caffeic acid, caffeine and glucose, commonly present in coffee, were evaluated as possible interfering agents. The interference was evaluated in terms of variation of the biosensor response for 1.99 μmol L^{-1} 5-CQA after the addition of 1.99 (1:1) and 19.6 (1:10) μmol L^{-1}, of each interfering compounds in phosphate buffer solution (pH 4.0) using the proposed biosensor. The biosensor response varied no more than 4.5% after the addition of 1.99 μmol L^{-1} of the interfering compounds, and no more than 6.3% after the addition of high concentrations (19.6 μmol L^{-1}) of the interfering compounds, thus indicating very low interference in the CGA determination by the biosensor device developed.

Brewed coffee beverages were directly analyzed by the laccase biosensor using SWV, and for comparative purposes, were also analyzed by a HPLC method (Vinson et al., 2019). The results of the content of 5-CQA in the brewed traditional and specialty coffees are presented in Table 2. As can be seen, the two methods yielded very similar results with relative errors lower than 6.42%, and the calculated F values also indicated that there was no difference in the variance of the results obtained by both methods. An analysis of Pearson's correlation (Fig. S2 A – supplementary material) showed r = 0.975 indicating high correlation between the results obtained by both methods of analysis. The paired t-test was also applied to these results, and demonstrated no significant difference between the means obtained by both methods (t = 0.598; p > 0.553, DF: 41). Finally, the analysis of a Bland-Altman plot (Fig. S2 B – supplementary material) showed high concordance between the CGA content achieved by the two methods, with all the results within the limits of agreement.

The CGA content measured as 5-CQA in the different types of brewed coffees was also evaluated. A t-test was applied to the CGA contents in the traditional and specialty coffees obtained by the laccase biosensor analyses. The results of the t-test indicate that the mean

contents of CGA in the traditional and specialty coffees were significantly different ($t = 4.911$; $p > 0.001$; DF: 40). In this study, we found that the content of CGA in the specialty-classified coffees (mean: 339 mg 100 mL^{-1} beverage) was greater than in the traditional coffees (268 mg 100 mL^{-1} beverage). These results were in close agreement to the CGA contents reported in various coffee-related products (Farah, 2012; Jeon et al., 2019), and slightly lower than those obtained in coffee under different brewing processes (Jeon et al., 2017).

Farah et al. (2006) found that a lower concentration of CGA is related to coffee beverages of high quality, as classified by sensorial analysis. On the otherhand, Clifford (1997) pointed-out that lower amounts of CGA can be responsible in avoiding the formation of potentially mutagenic products during the coffee roasting process, and that it can also minimize oxidative deterioration of the roasted coffee. But, according to Zanin et al. (2016) the quality of coffee cannot be attributed to CGA concentration alone since it presents a wide variation in coffees with similar degrees of roasting. Therefore, the results obtained in this work are attributable to the meticulous care taken in the processing of specialty coffees, i.e., the post-harvesting processes were more controlled. The roasting process is responsible for the degradation of many chemical products in coffees (Farah & Donangelo, 2006; Jeon et al., 2017), and in the specialty coffees, this step can be milder implying a lower degradation of compounds.

3.6. Discrimination between specialty and traditional coffees by square-wave voltammetric fingerprint and principal component analysis

The classification of specialty coffees is based upon sensorial analysis that demands a high degree of training of the baristas, who are subject to numerous interferences during the analyses. Chemical methods of analyzing, discriminating and classifying of these coffees can provide a highly reliable and fast process that is reproducible and, in this sense, a chemical fingerprint allied to chemometric tools can be applied for the discrimination of specialty from traditional coffees. The voltammetric fingerprint is defined as the characteristic of the voltammetric profile that distinguishes the specific metabolic set due to compositional chemical changes (Tsopelas, Konstantopoulos, & Kakoulidou, 2018). Principal component analysis (PCA) is an unsupervised chemometric method that reduces data by geometrically projecting them onto lower dimensions called principal components (PCs) in which discrimination patterns are encountered without prior knowledge of the samples, i.e., quality, origin, composition (Worley & Powers, 2013).

The voltammetric fingerprint approach was assessed as a potential tool for discrimination of specialty from traditional coffees. For this purpose, square-wave voltammograms obtained with the constructed biosensor for the coffees (Fig. 4A) were evaluated. As can be seen, no discriminating patterns could be identified in the voltammograms. Therefore, the voltammograms were subjected to PCA. The data matrix consisted on 42 rows (21 specialty and 21 traditional coffees) and 157 columns (potential sweep). The best results of triplicate analyses were obtained by the Savitzky-Golay smoothing technique with a 15-point window, second order polynomial, second derivative, and with mean-centered data.

It was found that the first two PCs accounted for 69.62% and 15.70% of the total variance, respectively (Fig. 4B) and a discriminating pattern was observed. The score plot of PC1 and PC2 showed that PC1 was responsible for the discrimination of specialty and traditional coffees, with the exception of specialty coffee S4 (Fig. 4B). Table 2 shows that S4 coffee has a lower concentration of 5-CQA among the specialty coffees examined. The fingerprints for specialty coffees are located in the negative region of PC1, while traditional coffees are located in the positive region of the same PC. From the loading plot (Fig. S3, supplementary material), the specialty coffees were associated with a potential of 0.43 V, whereas the traditional coffees correlated with a potential of 0.33 and 0.52 V. The potential of 0.43 V is the reduction

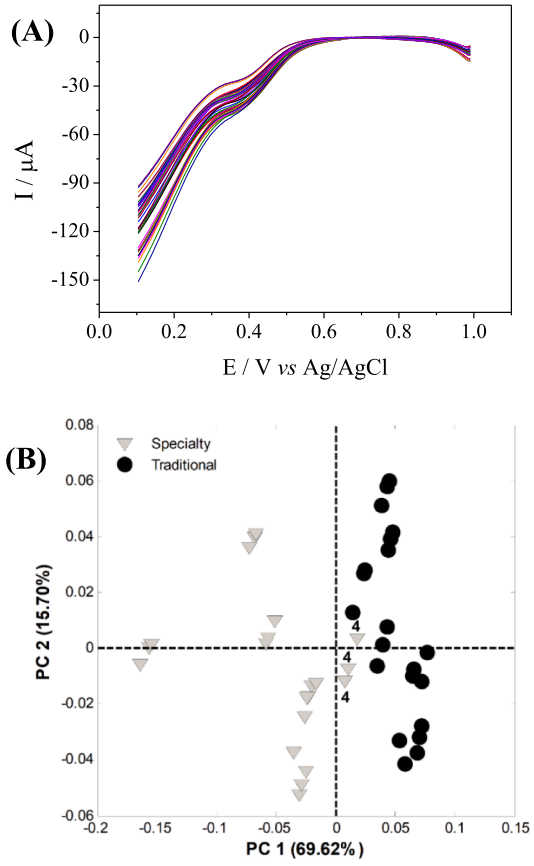

Fig. 4. (A) Square-wave voltammogram fingerprints of the brewed coffee samples analyzed by the laccase-based biosensor device that were subjected to PCA. (B) Score plot of the first two principal components of square-wave voltammetric fingerprints of specialty and traditional brewed coffee beverages showing their discrimination pattern.

potential of the 5-CQA (Fig. 3). Hierarchical cluster analysis by Ward's method of the square-wave voltammogram fingerprints also confirmed this separation of the PCA (Fig. S4, supplementary material). Voltammetric fingerprinting of specialty and traditional coffees obtained by the biosensor, in direct analysis, proved to be a powerful tool for quality control of these coffees based upon the relative abundance of the 5-CQA present. This tool can be associated with sensory analysis, and expanded for other similar complex matrices.

4. Conclusion

In this work, we presented a new architecture for constructing a biosensing device fabricated with PtNPs, BOT and laccase through using a statistical mixture design, and applied the biosensor to determine CGA (measured as 5-CQA) content in brewed traditional and specialty coffee beverages. Under the optimized experimental conditions, the laccase-based biosensor presented a linear response ($R^2 = 0.998$) for 5-CQA in the range of concentration 0.56–7.3 µmol L^{-1} with detection and quantification limits of 0.18 and 0.59 µmol L^{-1}, respectively. The biosensor device had a lifetime of 150 measurements, and a storage stability of 30 days, with a reduction of 6.59% of the initial response. The proposed biosensor was employed to analyze CGA in brewed coffees resulting in statistically similar results to those obtained by HPLC at the 95% confidence level. In addition, statistical analysis and PCA proved that the CGA content in the traditional and specialty coffees was significantly different. Thus, the proposed biosensor device composed of PtNPs, BOT and laccase can serve as a

selective voltammetric determination method of CGA for quality control of brewed coffee beverages.

CRediT authorship contribution statement

Carlos A.R. Salamanca-Neto: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Funding acquisition. **Gustavo G. Marcheafave:** Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Funding acquisition. **Jessica Scremin:** Methodology, Formal analysis, Investigation, Funding acquisition. **Eduardo C.M. Barbosa:** Resources, Writing - review & editing, Funding acquisition. **Pedro H.C. Camargo:** Resources, Writing - review & editing. **Robert F.H. Dekker:** Resources, Writing - review & editing. **Ieda S. Scarmínio:** Resources, Formal analysis, Software. **Aneli M. Barbosa-Dekker:** Conceptualization, Resources, Supervision, Funding acquisition. **Elen R. Sartori:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge financial support and scholarships from the Brazilian government funding agencies CNPq (grant number 408591/2018-8; E.R. Sartori), CAPES (Finance Code 001 (grant numbers 88882.448537/2019-01; C.A.R Salamanca-Neto, 88882.448533/2019-01; G.G. Marcheafave, and 88882.448536/2019-01; J. Scremin) and PROAP 2018), Fundação Araucária do Paraná (grant number 001/17-3765; E.R. Sartori, and proc. 45763 – Conv. 296/2015; A.M. Barbosa-Dekker), and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant number 2015/11452-5; E.C.M. Barbosa). The authors are also thankful to Dr. Maria L. Zeraik and Marcela B. Abreu MSc., Universidade Estadual de Londrina, for supplying the coffee samples used in this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126306.

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