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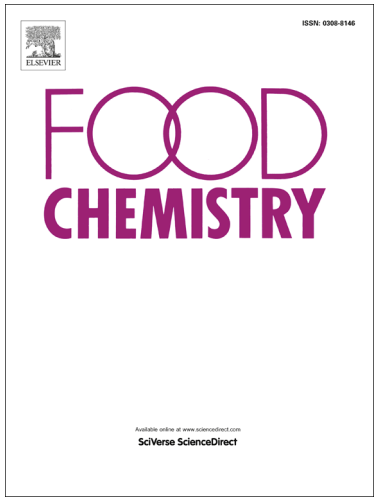

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Electroanalysis and laccase-based biosensor on the determination of phenolic content and antioxidant power of honey samples

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Abstract

Honey is a functional food widely consumed. Thus, the evaluation of honey samples to determine its phenolic content and antioxidant capacity (AOC) is relevant to determine its quality. Usually AOC is performed by spectrophotometric methods, which lacks reproducibility and practicality. In this context, the electroanalytical methods offer higher simplicity and accuracy. Hence, the aim of this work was to use of electroanalytical tools and laccase based biosensor on the evaluation of AOC and total phenol content (TPC) of honey samples from different countries. The antioxidant power established by electrochemical index presented good correlation with the spectrophotometric FRAP (Ferric Reducing Ability of Plasma) and DPPH (2,2-Diphenyl-1-Picrylhydrazyl) radical scavenging assays. Also, TPC results obtained by the biosensor agreed with the Folin-Ciocalteu (FC) assay. In addition to the semi quantitative results, the electroanalysis offered qualitative parameters, which were useful to indicate the nature of major phenolic compounds.

Keywords: Honey; Electrochemical index; Antioxidant power; Polyphenol; Natural antioxidants; Functional foods.

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1. Introduction

Honey is a complex natural food composed mainly of carbohydrates, water, traces of organic acids, enzymes, amino acids, pigments, pollen, wax and a myriad of minor components that can come directly from plants nectar, due to maturation process, as well as, be added by the bees (*Apis melliferas*) (Anklam, 1998; Silva et al., 2016).

The functional status of honey is related to the valuable quantity of natural antioxidants that come from the bee-collected pollen and other floral exudates (Schramm et al., 2003). Several of these antioxidants substances are secondary metabolites with high reducing power, such as the catechol-like polyphenols, *i.e.* flavonoids, anthocyanins, hydroxycinnamic acids, which are widely distributed in flowers, being responsible for their protection, color and attraction of pollinating insects (Sergiel, Pohl and Biesaga, 2014). The polyphenols are potent antioxidants that can reach more than 0.8% (w/w) in apicultural products (Krover and Hedegus, 2001). In turn, dietary intake of polyphenol compounds provides protection against free radicals and reactive oxygen species (ROS), which are harmful compounds involved in the genesis of many diseases, including cancer and acceleration of the aging process (Krover and Hedegus, 2001; Sergiel, Pohl and Biesaga, 2014). Hence, the consumption of food with high antioxidant content is an irrevocable trend, in which the honey can act as the main alternative for sweetening products (Anklam, 1998; Krover and Hedegus, 2001; Schramm et al., 2003; Escriche et al., 2011, 2014; Sergiel, Pohl and Biesaga, 2014; Fechner et al., 2016; Silva et al., 2016).

The apiculture is commonly based on native forests or citrus and eucalyptus plantations, where the flora allied to edaphoclimatic factors dictates the honey variety, the inherent polyphenol content and, thereby, its antioxidant power (Anklam, 1998; Escriche et al., 2011, 2014; Fechner et al., 2016; Silva et al., 2016).

The evaluation of total phenolic content (TPC) and antioxidant capacity (AOC) of honey samples may aid in the elucidation of their authenticity (Escriche et al., 2011,2014; Sergiel, Pohl and Biesaga, 2014; Silva et al., 2016) and must be included as quality attributes for this functional food (Alves et al., 2013; Escudero et al., 2013; Escriche et al., 2014). The TPC and AOC of foods and natural products have been mostly measured by traditional spectrophotometric methods, *i.e.* DPPH, ABTS and Folin-Ciocalteu assays (Frankel et al., 1998; Malaver and Vit, 2007; Escudero et al., 2013; Gorjanović et al., 2013; Pérez, Rodriguez- Bueno-Costa et al., 2016; Clementino et al. 2016).

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Due to the inherent redox correlation, the electroanalytical methods emerge as a viable and foresighted alternative, combining simplicity and lower cost and providing qualitative and quantitative data (Reis et al., 2009; Gorjanović et al., 2013; Clementino et al., 2016). Thus, the electrochemical index, firstly proposed by Escarpa research group, (Blasco, González and Escarpa, 2004; Blasco et al., 2005) and then reinforced and revisited by our research group, has proved to be a useful tool to express the antioxidant capacity of different complexes matrices, such as wine (Lino et al., 2014), coffee (Oliveira-Neto et al., 2016), and red fruits extracts (Macedo et al., 2017). Moreover, the selectivity of electrochemical sensors can be adjusted and improved. For instance, the fusion between electrochemical transducers with redox enzymes offers increased sensitivity and a suitable selectivity (Gil et al., 2009; Gil and Rebelo, 2010; Campanella, Bonanni and Tomassetti, 2013; Rodríguez-Delgado et al., 2015). Among redox enzymes, laccases which are easy to obtain and present good stability, are useful to determine TPC and AOC, since they are recognized for their high and wide activity against many phenolic compounds (Gil et al., 2009; Gil and Rebelo, 2010; Rodríguez-Delgado et al., 2015).

Hence, the aim of this work was the application of voltammetric techniques and laccase-based biosensor (LbB) on the evaluation of antioxidant power and profile of honey samples, evidencing the correlations between electrochemical and spectrophotometric approaches. The main electrochemical parameters, namely, peak current (I_{pa}) and peak potential (E_{pa}) were obtained by differential pulse voltammetry (DPV) at carbon paste electrode, and correlated with DPPH and FRAP assays, in order to ascertain the AOC of honey samples. Meanwhile, the (LbB) was proposed as a tool to reach the TPC, and then compared with FC traditional method.

2. Experimental

2.1 Reagents and samples

Methanol, ethanol, hydrochloric acid (HCl), Iron(III) chloride ($FeCl_3$), Iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$), potassium phosphate, sodium acetate, sodium carbonate and potassium persulfate were obtained from Vetec (Rio de Janeiro, Brazil), all analytical grade. ABTS (2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)), DPPH (1,1-diphenyl-2-picrylhydrazyl), Folin-Ciocalteau reagent, 2,4,6-Tripyridyl-s-Triazine (TPTZ), gallic acid, cathecol, rutin, hesperidin, were purchased from Sigma

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Chemical Co. (St. Louis, MO, USA). All electrolyte solutions were prepared using Milli-Q water (conductivity ≤ 0.1 μS cm^{-1}) (Millipore S.A., Molsheim, France).

In order to achieve greater variability, the honey samples purchased from four countries, namely Brazil (BR samples 1 to BR 18), United States (US sample 19), Portugal (PT samples 20 and 21), and New Zealand (NZ samples 22 to 24), were acquired from different supermarkets and had color scales ranging from white to dark amber.

2.2 Spectrophotometric Determinations AOC and TPC

The spectrophotometric measurements were carried out in a UV-visible spectrophotometer Q798U2VS (Quimis Aparelhos Científicos, São Paulo, Brazil).

2.2.1 DPPH AOC Assay

The radical Scavenging activity of samples was evaluated by measuring their decolorizing effect on stable DPPH radical as described by Brand-Williams (1995) with modifications, as follows. Briefly, 2.5 mg of DPPH reagent was diluted in 100 mL of methanol:water (8:2 v/v). To 2.5 mL of this solution (stock solution) an aliquot of 0.5 mL of the same solvent system till the measured absorbance (A) reached 0.7 (λ 517 nm) was added. The same volume (0.5 mL) of gallic acid standard solutions (0 to 13.3 μmol L^{-1}) and honey samples properly diluted (0.2 g mL^{-1}) were added to 2.5 mL of stock solution, and kept in dark at 25 °C for 5 minutes.

The results were expressed by DPPH decolorizing effect in percentage (%), according to the equation 1:

$$\mathbf{AOC}\ (\%) = \left(\frac{\mathbf{A_{control} - A_{antioxidant\ system}}}{\mathbf{A_{control}}}\right) \mathbf{x\ 100} \qquad (1)$$

The antioxidant system was the honey sample or gallic acid standard, also used to express the AOC by means of gallic acid equivalents (GAE / μmol L^{-1}).

2.2.2 FRAP AOC assay

The FRAP assay procedure is based in the Fe^{3+} reduction to Fe^{2+}. The FRAP reagent, prepared at beginning of the analysis, consisted of a mixture of 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mmol L^{-1}), HCl (40 mmol L^{-1}), $FeCl_3$ (20 mmol L^{-1}) and sodium acetate buffer (0.3 mol L^{-1}; pH 3.6) in the following proportion, 1:1:1:10, respectively. 200 μL aliquots of the honey solution (0.2 g mL^{-1}) were injected in 1.8 mL

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FRAP reagent and left in a water bath at 37 ºC for 10 minutes. Then, absorbance was measured at 593 nm. $FeSO_4.7H_2O$ aqueous solution (100 to 1000 µmol L^{-1}) was used in the calibration curve, and the results were expressed as µmols Fe II equivalents per honey gram (Benzie and Strain, 1996; Bertoncelj et al., 2011; Escarpa, 2012; Gil et al., 2013/2014; Gomes et al., 2016).

2.2.3 Folin-Ciocalteu (FC) assay

The TPC was determined by Folin-Ciocalteu spectrophotometric method (Singleton, Orthofer and Lamuela-Raventós, 1999). In brief, 100 µL honey aqueous solution (0.2 g mL^{-1}) aliquots were mixed with 2 mL distilled water and 0.5 mL Folin-Ciocalteu reagent in a 10 mL volumetric flask. After 2 minutes, 1.5 mL sodium carbonate (20% w/v) was added, the solution was stirred and the volume measured with distilled water, after homogenization. The solution was centrifuged and kept 1 hour in the dark, at room temperature. Then, absorbance was measured at 765 nm. TPC was calculated by calibration curve (2.5-10 µg mL^{-1}) and expressed in gallic acid equivalent mg per honey gram.

2.3 Electroanalytical Approaches for AOC and TPC determinations

The voltammetric tests were conducted on a Potentiostat/galvanostat µAutolab III® software integrated with GPES 4.9®. The electrochemical cell used has a volume of 2 mL and a system of three electrodes. The Carbon Paste Electrode (CPE, d = 1.5 mm), a $Ag/AgCl/KCl_{sat.}$ and a platinum wire were the working, reference and counter electrodes, respectively, herein used for electrochemical characterization and electrochemical index determination.

The CPE was polished using soft paper before each electrochemical experiment. After polishing, it was placed in a buffer supporting electrolyte, in which around 10 voltammograms were recorded in order to reach the steady state baseline.

The instrumental parameters for differential pulse voltammetry (DPV) were: scan rate of 10 mV s^{-1}, pulse amplitude 50 mV. For square wave voltammetry (SWV) were: pulse 50 mV, frequency 50 Hz and potential increment 2 mV, corresponding to an effective scan rate of 100 $mV.s^{-1}$. The pH measurements were carried out with a Quimis Q400AS pH-meter with a Quimis QA 338 combined glass electrode. All measurements were performed at room temperature.

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2.3.1 Electrochemical Index on the AOC determination

An electrochemical index (EI) was proposed considering the main voltammetric parameters, peak potential (E_{pa}) and peak current (I_{pa}). Thus, since the lower the potential (thermodynamic parameter), the higher the electron donor ability, as well as the higher the peak current (kinetic parameter), the higher the amount of electroactive species, the EI was calculated by using the equation 2:

$$EI = \sum_{i=1}^{n} \frac{Ipai}{Epai} \qquad (2)$$

Where I_{pa} and E_{pa} correspond to current and potential values for each major anodic peak observed in the DPV voltammograms; n stands for number of major anodic peaks (Blasco, González and Escarpa, 2004; Blasco et al., 2005; Lino et al., 2014; Gomes et al., 2016; Oliveira-Neto et al., 2016).

2.3.2 Laccase-based Biosensor on TPC determination

A laccase-based modified carbon paste biosensor (LbB) was used as working electrode in order to estimate the TPC. The LbB was prepared accordingly to previous work (Gil et al., 2013/2014). Briefly, 500 µl of partially purified crude extract of laccase (from *Pycnoporus sanguineus*) was added to 15 mg of organo-functionalized silica and left to dry at room temperature, and then, 65 mg of graphite was added and homogenized in a mortar with pestle for 15 minutes, then 35 mg of mineral oil were added to obtain homogeneous paste. This biosensor exhibited greater stability and activity than the one prepared with carbon paste only (Gil et al., 2013/2014).

2.3.3 Electrochemical Data Treatment

The data obtained were baseline-corrected using the moving average application included in GPES 4.9® software. This treatment aimed to improve the visualization and identification of peaks. The plot of the voltammetric curves for final presentation in this study was drawn using Origin Pro 8® software. The data treatments were performed with care to avoid introducing any artifacts.

2.4 Multivariate Calibration and Statistical analysis

Statistical analysis was performed using XLSTAT Version 2014.5.03. The statistical confidence level of 95% was employed for Sperman principal component

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analysis (PCAs) and hierarchical cluster analysis (HCA) (Antunes et al., 2015). Was adopted a similarity metric of Spearman correlation coefficient distance between observations, agglomeration method of weighted pair-group average. Finally, the Cross-validation was used with 3 and 10 folds.

3. Results and Discussion

The antioxidant activity of each honey sample was evaluated by different methods (Table 1). However, due to the complexity of these natural products, in which the chemical composition varies according to flora and edaphoclimatic features of their place of origin, it is reasonable to expect different interfering effects. Therefore, the use of techniques of dissimilar analytical principles is imperative to improve assertiveness (Anklam, 1998; Bertoncelj et al., 2011; Gorjanović, et al., 2013; Escriche et al., 2014).

3.1 Spectrophotometric assays

The TPC and DPPH assays were performed by spectrophotometric methods. The TPC varied from 6.15 a 149.70 µg of gallic acid equivalents (GAE) per gram of honey. The average results for Brazilian and Portuguese samples were quite close, being of 38.15 and 37.86 µg GAE.g^{-1}, respectively. The New Zealander pool samples presented a lower average value of 26.45 µg GAE.g^{-1}. Gorjanović et al. (2013) have found values that ranged from 94 to 620 µg GAE.g^{-1}. Despite the perspectives (Bertoncelj et al., 2011; Gorjanović et al., 2013), no correlation was observed between color intensity and TPC results (Table 1).

INSERT TABLE 1

The concentration to decolorize the DPPH radical ranged from 278.8 to 2.44 mg g^{-1}. The minimum required amount of honey sample was obtained for the Portuguese sample PT 21, whereas the higher was observed for the Brazilian sample BR 12, both of amber color. The EC_{50} (mg g^{-1}) average obtained for Brazilian honey samples against DPPH assay was of 51.63 mg g^{-1} (Table 1).

Finally, the samples were also evaluated by FRAP AOC assay, a colorimetric method that resembles the FC by using metal probe. Indeed, a slight linear correlation of 0.6144 was observed between FRAP and FC methods. This correlation was observed

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too in PCAs analyses where Spearman correlation was 0.8896. The FRAP found values varied from 10.83 to 407.45 μmol L^{-1} Fe^{2+} g^{-1} of honey (Table 1).

Therefore, all spectrophotometric methods presented a huge variability for antioxidant determinations. This variability may be due to the fact that many chemicals can absorb visible light at the same wavelength, including natural compounds, caramel and products of Maillard reactions. An alternative for colorimetric methods is the use of electrochemical index concept (Escarpa, 2012; Lino et al., 2014; Clementino et al., 2016; Oliveira-Neto et al. 2016). It takes into account the peak potentials, E_{pa}, and peak currents I_{pa} in order to express the thermodynamic reducing power and kinetic/concentration of antioxidant species present in samples. The higher the E_{pa} values, the lower the antioxidant power. In turn, the I_{pa}, accordingly to Faraday law and kinetic of electron transfer processes has a quantitative meaning. The advantages of electrochemical methods are very well established and, indeed, it has been reviewed in high impact journals (Escarpa, 2012; Hoyos-Arbeláez et al. 2017).

3.2 EI concept

The electrochemical index (EI = μA mV^{-1}) of samples was estimated by the sum of the ratios, I_{pa}/E_{pa} (equation 1), of each anodic peak obtained in DP voltammograms for the honey samples (Figure 1).

INSERT FIGURE 1

The EI values ranged from 12.97 to 99.14 μA mV^{-1} g^{-1}, presenting lower amplitude variation than the colorimetric assays (Table 1). Nevertheless, the voltammetric profiles of different samples differed greatly. Indeed, the honey samples presented 2 or 3 anodic peaks, the first at c.a. 0.2 V, the second at c.a. 0.5 V and the third occurring nearby 0.8 V (Figures 1 and 2) *vs* $Ag/AgCl/KCl_{sat.}$ The first anodic process, peak 1a, is related to species with highest electron donor character, whereas the third with the lowest. For instance, in NZ samples the peak 1a presented high intensity when compared to peak 3a, but the peak currents is lower. It can be inferred that the overall phenolic content of these samples is lower, which leads to low EI values (Figure 2).

INSERT FIGURE 2

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3.3 Statistical and Chemometric analysis of intercorrelations

Initially, an exploratory analysis of the dataset was done, the analysis of the main components of Spearman was performed with the objective of verifying a possible separation of honey samples from different regions and / or countries using the results calculated for total phenols. Data was self-scaled and four principal components variance were enough to construct the PCAs model, as 91.54% explained of the variance of data F1-F2 (Figure 3A).

The PCA is one of the most used statistical procedure multivariate data analysis. It uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables. When PCA is done with heterogeneous units, it is necessary use Spearman's correlation coefficient, which assesses monotonic relationships (whether linear or not). The HCA is an iterative classification method whose principle is to build a hierarchy of clusters by agglomerative (bottom up) or divisive (top down) strategies.

The Figure 3 shows that there was no clear separation between the regions of origin of the samples (countries). However, the distribution of scores for the components 1 and 3 shows an ascending order in the antioxidant capacity of the samples (left to right) while the samples 4, 13 16 and 17 have the largest capacity, and the sample 12 has the smallest. This behavior is defined by the principal component # 1 that contains 74.01% of data variance (Figure 3A).

INSERT FIGURE 3

Regarding the contributions of variables, the influence from DPPH is inversely proportional to EI with a - 0.75 monotonic relationships value. On the other hand FRAP and FC, as expected, have a directly and strongly correlated Spearman (0.89). This can be observed in the behavior of loadings (Figure 3A) and in HCA (Figure 3B). This last figure shows the Spearman`s correlation similarity between the variables EI and Total Phenols and also with DPPH. The variable FRAP was separated from the others.

These correlations are in accordance with data already described in the literature, thus proving the efficacy of electroanalytical tools to designate antioxidant profile in different matrices (Blasco et al., 2005; Lino et al, 2014; Oliveira-Neto et al., 2016; Macedo et al., 2017).

3.4 Laccase-based Biosensor (LbB) on the TPC evaluation

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The LbB was applied in order to estimate the TPC content in the selected honey samples investigated. The biosensor presented high activity in mild acid pH and the response time, which corresponds to the time for enzymatic oxidation of phenolic compounds, was lower than 30 s but progressively increased up to 240 s, when they reached a plateau, remaining practically stable. Then the biochemically oxidized phenolic compounds were electrochemically reduced at LbB electrode surface. The corresponding cathodic peak currents were used to express the TPC value and presented good correlation (r) and small error (R^2) with FC values (Table 2 and Figure 4).

INSERT FIGURE 4

INSERT TABLE 2

4. Conclusions

The electroanalytical and spectrophotometric approaches demonstrated good correlation for AOC determination. Likewise, the *polyphenoloxidase* based biosensor (LbB) exhibited strong correlation with the traditional Folin-Ciocalteu method. Despite the much lower proportion, the phenolic composition had a greater contribution on the AOC than reducing sugars. Therefore, the honey samples presented reasonable and variable antioxidant activity, which was dictated mainly by sample origins than the color intensities.

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Compliance with Ethical Standards

Conflict of Interest Isaac Yves Lopes Macedo declares that he has no conflict of interest. Jerônimo Raimundo de Oliveira Neto declares that he has no conflict of interest. German Sanz Lobón declares that he has no conflict of interest. Luane Ferreira Garcia declares that she has no conflict of interest. Eric Gil declares that he has no

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conflict of interest. Stefani Rezende declares that she has no conflict of interest. Telma Garcia declares that she has no conflict of interest. Virgínia Farias Alves declares that she has no conflict of interest. Mariângela Santiago declares that she has no conflict of interest. Ieda Torres declares that she has no conflict of interest. Fernando Schmidt declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent was not applicable.

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Legends of Tables and Figures

Tables

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Table 2. Total phenol content obtained by FC and LbB methods.

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Figure 2. Baseline corrected DP voltammograms obtained for different 10% BR (A), PT (B) and 20% NZ (C) samples solutions in pH 6.0 0.1 mol L^{-1} PBS at carbon paste electrode.

Figure 3. Statistical correlations: A) Biplot distribution of PCA scores for the honey samples from the results of AOC (DPPH, FRAP and EI) and TPC (FC) assays. The loadings are represented in diagonal lines. B) Hierarchical Clustering Analysis – HCA.

Figure 4: A) Effect of equilibrium time on LbB response. B) Average DP voltammograms obtained for different 10% honey samples, BR 13 (—),BR 17 (- - -), NZ 22 (- •-), US 19 (- ••-), and blank (•••). All in 0.1 mol L^{-1} PBS, pH 6.0.Other parameters included a pulse width of 5 mV, pulse amplitude of 50 mV, scan rate of 10 $mV.s^{-1}$.

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Table1. Results of AOC (DPPH, FRAP and EI) and TPC (FC) assays for honey samples of various origins, acquired from different suppliers.

Samples*	**FC** µg (GAE).g^{-1}	**DPPH**mg.g^{-1}	**FRAP** µM(Fe^{+2}).g^{-1}	**EI** µA/mV.g^{-1}
BR 1[pw]	19.08	48.63	28.90	**12.97**
BR 2[pw]	27.83	60.61	42.16	18.92
BR 3[pw]	25.16	134.90	38.13	17.11
BR 4[pw]	20.20	69.70	28.78	44.38
BR 5[pw]	12.66	17.07	18.04	27.82
BR 6[la]	9.16	82.00	13.05	20.13
BR 7[la]	10.27	41.00	14.64	22.57
BR 8[la]	15.12	30.53	21.54	33.22
BR 9[a]	28.45	18.26	50.12	79.13
BR 10[a]	**6.15**	16.40	**10.83**	17.1
BR 11[a]	23.05	33.19	40.60	64.1
BR 12[a]	30.64	**278.80**	44.28	17.15
BR 13[a]	**149.70**	6.57	216.36	83.8
BR 14[da]	107.12	6.52	154.81	59.96
BR 15[da]	39.43	60.61	56.98	22.07
BR 16[da]	63.08	7.88	**407.45**	73.98
BR 17[da]	71.64	9.46	379.12	**99.14**
BR 18[da]	27.88	7.30	147.57	38.59
US 19[la]	26.70	11.91	198.95	95.62
PT 20[la]	25.33	53.87	25.86	28.45
PT 21[a]	50.39	**2.44**	264.95	78,24
NZ 22[a]	34.14	15.18	168.12	32.15
NZ 23[la]	21.01	19.26	61.65	35.28
NZ 24[la]	24.20	18.51	39.56	22.64

*RSD<1,28 %; legend: pale white[pw], light amber[la], amber[a], dark amber[da].

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Table 2. Total phenol content obtained by FC and LbB methods.

Honey Samples	FC (µg (GAE).g^{-1})	TPC-LbB (µg (GAE).g^{-1})
BR 13	149.70	140.88
BR 17	71.64	68.98
NZ 22	34.14	28.02
US 19	26.70	17.25

*FC:LbB correlations: Linear correlation (r) = 0.933, $R^2 < 0.99$.

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Highlights

- Antioxidant power analyses of diverse honey samples
- Electroanalytical method as alternative to conventional and colorimetric analysis
- Laccase-based biosensor on the determination of total phenol content in honey

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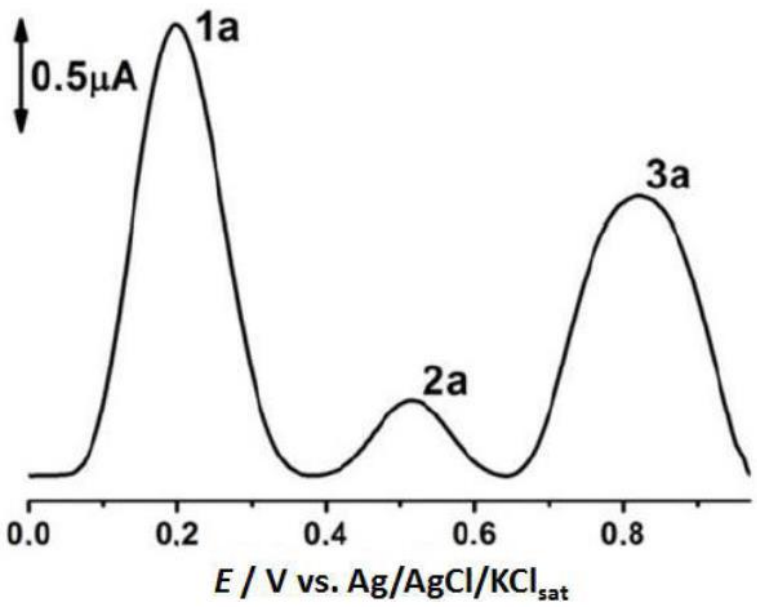

Figure 1. Baseline corrected DP voltammograms obtained for 20% US 19 sample solution in pH 6.0 0.1 mol L^{-1} PBS at carbon paste electrode.

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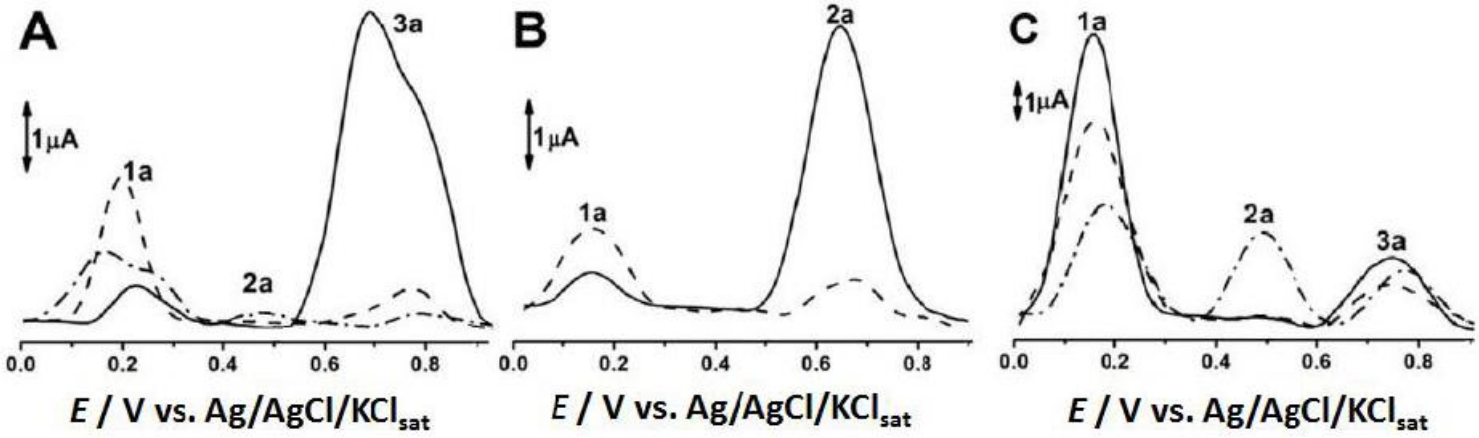

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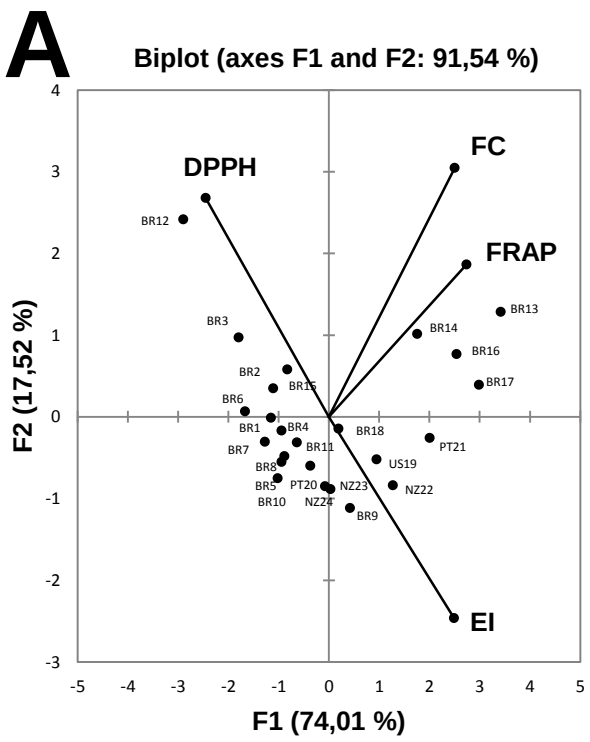

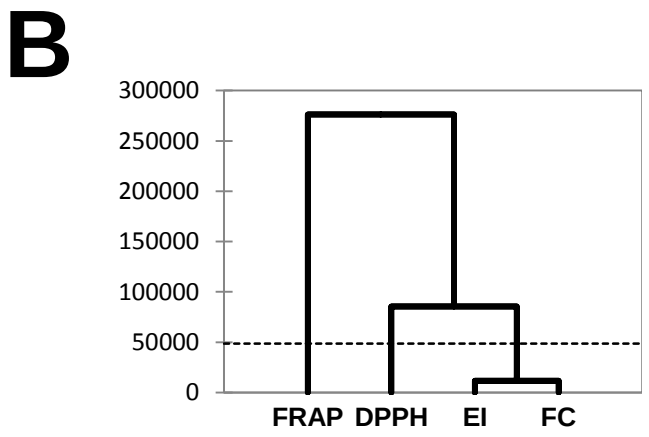

Variables	EI	FC	DPPH	FRAP
EI	1			
FC	0,5078	1		
DPPH	-0,7519	-0,4910	1	
FRAP	0,6470	0,8896	-0,6232	1

Figure 3. Statistical correlations: A) Biplot distribution of PCA scores for the honey samples from the results of AOC (DPPH, FRAP and EI) and TPC (FC) assays. The loadings are represented in diagonal lines. B) Hierarchical Clustering Analysis – HCA.

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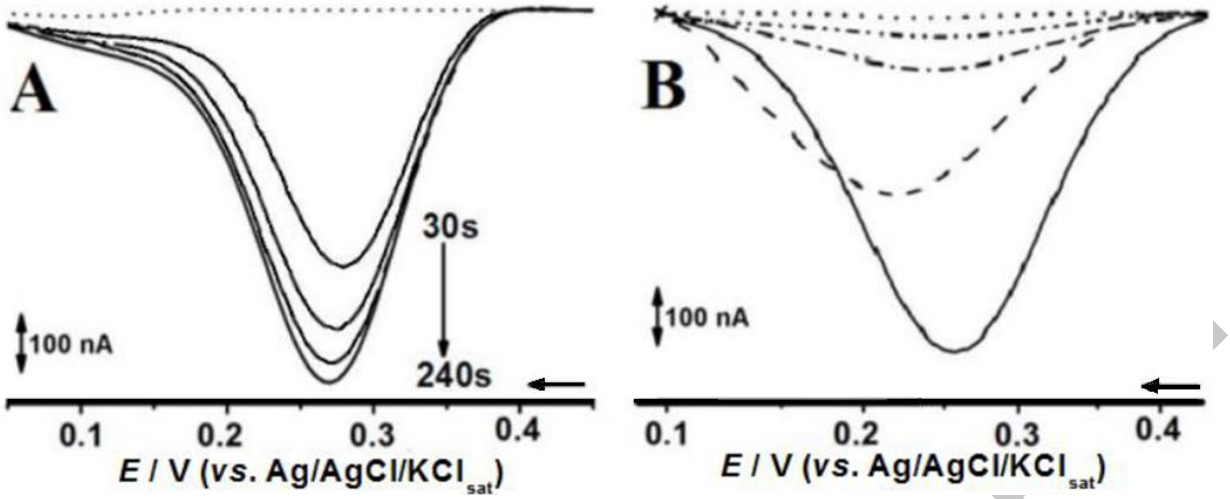

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