CHAPTER 6

Electrochemical Sensors for Assessing Antioxidant Capacity of Bee Products

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Abstract: This chapter is focused on the application of electrochemical techniques (*e.g.*, sensors and biosensors), as the predominant methodology, to the quantification of individual or total phenolic compounds, either in standard solutions or in real matrices (*e.g.*, plants, fruits and beverages) and their capability for assessing antioxidant activity/capacity. Specially, the potential application to evaluate antioxidant capacity of bee-hives products (*e.g.*, propolis, honey) is addressed. Finally, the voltammetric behavior of Portuguese monofloral honeys is discussed for the first time, taking into account the expected effects of honey color and floral origin.

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Also, a possible relation with the expected antioxidant capacity of honeys is discussed, considering their floral origin. Works describing the use of electrochemical detection imbibed on liquid chromatographic or capillary electrophoretic configurations among other analytical methods will not be focused in this review, although their undoubtedly potentials and proved applications.

Keywords: Antioxidant activity, Bee products, Cyclic voltammetry, Differential pulse voltammetry, Electrochemical techniques, Honey, Phenolic compounds, Pollen, Propolis, Square wave voltammetry.

1. INTRODUCTION

Honey, propolis and pollen are plant-derived products and a source of polyphenolic compounds. Honey has a high content of sugars and small amounts of minerals, proteins and other constituents namely flavonoids, phenolic acids, enzymes, amino acids and vitamins [1, 2]. In the case of honey, the total phenolic content appears to be strongly correlated with the antioxidant activity, being the highest values found in darker honeys [1, 3 - 6]. Propolis is a resinous material, which pharmaceutical properties are well known and attributed to the high contents in polyphenols [7] (flavonoids, phenolic acids and their esters), as well as terpenoids, steroids and amino acids [8]. Bee pollen also contains considerable amounts of phytochemicals and nutrients, being rich in carotenoids, flavonoids and phytosterols [9]. The floral and geographical origins affect greatly the quantity and composition of polyphenol compounds in these bee's products, which reflects on the antioxidant capacity of each product [1, 8].

Electroanalytical methods are well-known tools used to study chemical and biological systems, allowing evaluating the antioxidant capacity of compounds that act as reducing agents, like phenolic compounds, which are easily oxidized on the surface of electrodes. These methods have several advantages when compared to other more traditional techniques (for example, spectrophotometric methods) since, they are simple methodologies, have low detection limits, good selectivity, reduced time of analysis, low consumption of reagents, having an overall lower environmental impact. Since, the electrochemical signals are due to the presence of analytes with electrical properties (antioxidants), it is not necessary to generate or use oxidized species. Among the electroanalytical techniques, three

voltammetric methods have been particularly reported as fast screening tools for assessing the composition quality and bioactivity of samples: cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square-wave voltammetry (SWV). These techniques use electrolysis conditions to study the phenomena occurring between the electrode surface and the thin solution layer in contact, differing in the way that potential is applied [10 - 13]. They allow to obtain quantitative and qualitative information of chemical species during the electrolysis using the current-potential curve generated (*i.e.*, the voltammograms).

In the literature, some works report the direct application of electrochemical techniques for bioactivity assessment and/or quality evaluation of bee-hive products, being mainly focused in honey and propolis analysis. Indeed, to the best of the authors' knowledge there is no study concerning pollen evaluation. Despite not being widespread used [14], these analytical techniques are an attractive approach to characterize compounds that act as reducing agents in natural products, being envisaged an increase of their application in a near future for bee products analysis.

2. VOLTAMMETRIC TECHNIQUES: GENERAL CONCEPTS

The voltammetric techniques that use electrochemical cells are based on two or three electrodes, immersed in a solution, which allows the movement of ions by charge transfer (electrolytes). The electrodes (metals or semiconductors, solid or liquid) allow the charge transfer through the electrons movement.

2.1. Electrochemical Cells

The electrochemical cells can be galvanic or electrolytic cells. In the galvanic cells, the reactions occur spontaneously in the electrodes converting the energy generated in a chemical reaction into electrical energy. Applying a potential to the cell beyond the potential of the reversible reaction, the reaction direction is changed, being possible the conversion of electrical energy into chemical energy. In these conditions, it is an electrolytic cell. The electrolytic cells allow studying the reduction (electron capture) and oxidation (release of electrons) phenomena, in general, under the action of an external controlled potential, enabling to control the reaction's direction and their intensity. So, accurate information about the

oxidation-reduction reactions (for example, on the concentration, thermodynamics, kinetics and reaction mechanisms) can be obtained [10 - 13].

In the electrochemical cell, the electrolyte solution (supporting electrolyte) reduces the medium resistance, eliminating the migration current and, depending on the analysis, keeps constant the solution pH (buffer solution). A three-electrode cell (Fig. **1**) should be used when the product between the current and the resistance component ($i \times R$) is high. Usually, there is a reference electrode (RE), a counter electrode (CE) and a working electrode (WE). The WE corresponds to an inert electrode and can be a metal (mercury, platinum or gold), a glassy carbon or a carbon paste. The WE's size, structure and material depend on the stability and selectivity of the WE surface towards the compound to be analyzed. The CE can be of any type (usually platinum), should be a good conductor and must not interfere with the reactions occurring in the solution. The RE may be a saturated calomel (Hg/Hg_2Cl_2) electrode or a silver/silver chloride (Ag/AgCl) electrode.

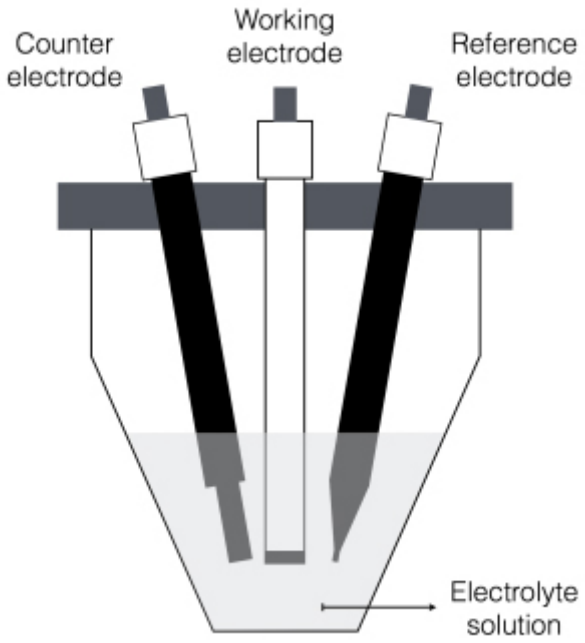

Fig. (1). Three electrodes typical electrochemical cell for voltammetry assays: working electrode (WE); counter electrode (CE); reference electrode (RE).

In this system, the current flows between the WE (usually, it has a small surface to quickly achieve the potential imposed on it) and the CE (the driving current from the WE). The potential is measured between the WE and the RE, which should be located as close as possible to the WE to reduce the resistance between them. Potentiostats are used in these methods, allowing the measurement of the applied potential (using high input impedance) and of the resulting current. This allows the passage of current through the RE, being the potential kept at a constant value. The potential and current are measured simultaneously being usually the recorded data visualized as a voltammogram (*i.e.*, the plot of current *vs*. potential scan).

As mentioned earlier, CV is one of the three most common electrochemical methods used for assessing antioxidant capacity of single- or multi-components of natural products, as well as for assessment of the total phenolic contents of natural samples.

2.2. Cyclic Voltammetry

The CV is a technique for acquiring quality information on the redox states, the oxidation state stability and electron transfer kinetics. In this technique, the potential is applied in two directions, namely in the form of a triangular wave, while the current is monitored. Fig. (**2A**) shows the potential parameters set over time, for a CV analysis and Fig. (**2B**) shows a typical cyclic voltammogram for a reversible redox process, being indicated the parameters that can be drawn from the chart: the cathodic peak potential (E_{cp}); the anodic peak potential (E_{ap}); the cathodic peak current (i_{cp}); and the anodic peak current (i_{ap}).

The analysis generates voltammograms where both the oxidation (anodic) and the reduction (cathodic) waves are plotted. In the absence of electroactive species, the electrochemical cell functions as a capacitor, so cations and anions migrate to the cathode and anode respectively, without an effective charge transfer, resulting in a voltage-current curve (voltammogram) without peaks. The presence of electroactive compounds in the interface between the solution thin layer and the electrode surface, leads to the appearance of current peaks, generated at their reduction and/or oxidation potentials, for the analyzed compound.

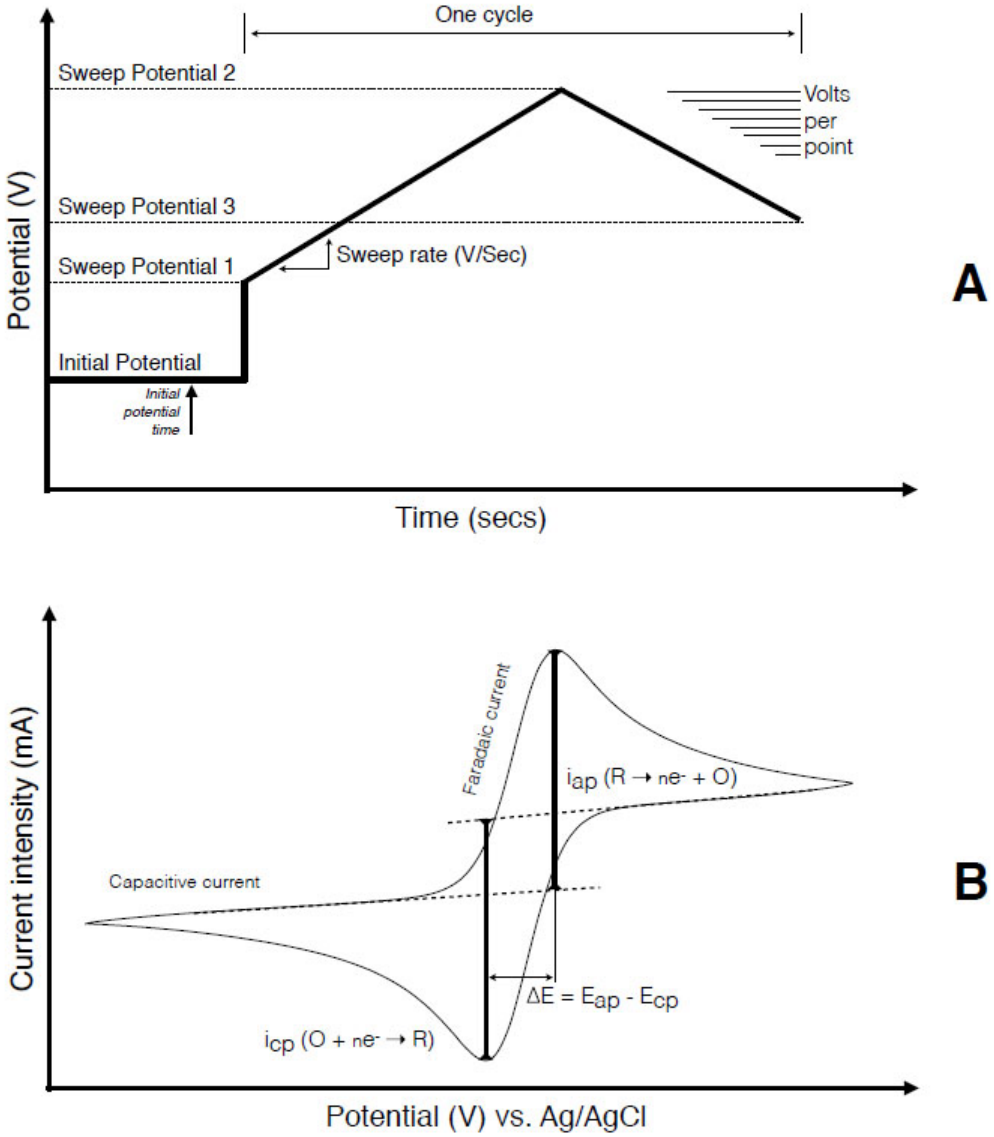

Fig. (2). CV analysis: scheme of the potential parameters to be set (**A**) and typical voltammogram (**B**).

For reversible redox processes, when the potential is reversed the newly oxidized species are reduced at the electrode interface, achieving the original state if the electrode reaches the polarization level at a sufficiently negative potential. In

reversible processes, the difference between the anodic and cathodic potentials is zero ($\Delta E = E_{cp} – E_{ap} = 0$) and the ratio between the reduction and oxidation current peaks is equal to 1 ($i_{ap} / i_{cp} = 1$). In some cases, the cyclic voltammograms show a quasi-reversible process, when differences from those values are observed. If an oxidized or reduced compound does not regenerate completely, by reversing the potential applied to the electrode, the redox process is irreversible [11]. Besides this qualitative information, if the peak current is proportional to the analyte's concentration, a calibration curve can be established enabling quantifying the redox active compounds present in the sample.

Overall, CV is commonly used to characterize the redox system [15 - 18], being a non-destructive electrochemical analytical technique with good sensitivity. In general, when CV is applied for analyzing natural products extracts, a voltage scanning is applied at the WE and the current observed due to the oxidation of an antioxidant compound (reducing agents that are able to donate an electron) is measured. For single compound analysis, the maximum current response at the anodic peak is proportional to its concentration but, when analyzing an extract containing a mixture of compounds, the area under the curve shows a better correlation with the total antioxidant capacity [16]. In antioxidant properties studies, a standard compound (for instance, caffeic acid), with a similar chemical structure as the target compounds, is used to establish a calibration curve, allowing quantifying the total phenolic content and the related antioxidant activity of the biological samples [19]. Also, it allows a comparison with the results from antioxidant spectrophotometric assays (for instance, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ferrous chelation assays). These general procedures are also used in DPV and SWV.

2.3. Differential Pulse Voltammetry

The DPV is a pulse voltammetry technique that has the capacity to discriminate charging (capacitance) current resulting in a more selective and sensitive technique towards oxidation or reduction currents (faradaic currents) than conventional voltammetry. This technique measures the difference of the current before (i(1)) and after a small potential pulse (i(2)) is applied, with amplitudes between 10 and 100 mV, for several milliseconds, superimposed on an applied

linear potential sweep (potential is changing linearly with time) [11, 12]. Fig. (**3A**) shows the potential parameters usually used to carry out a typical DPV analysis, being an example of voltammogram shown in Fig. (**3B**).

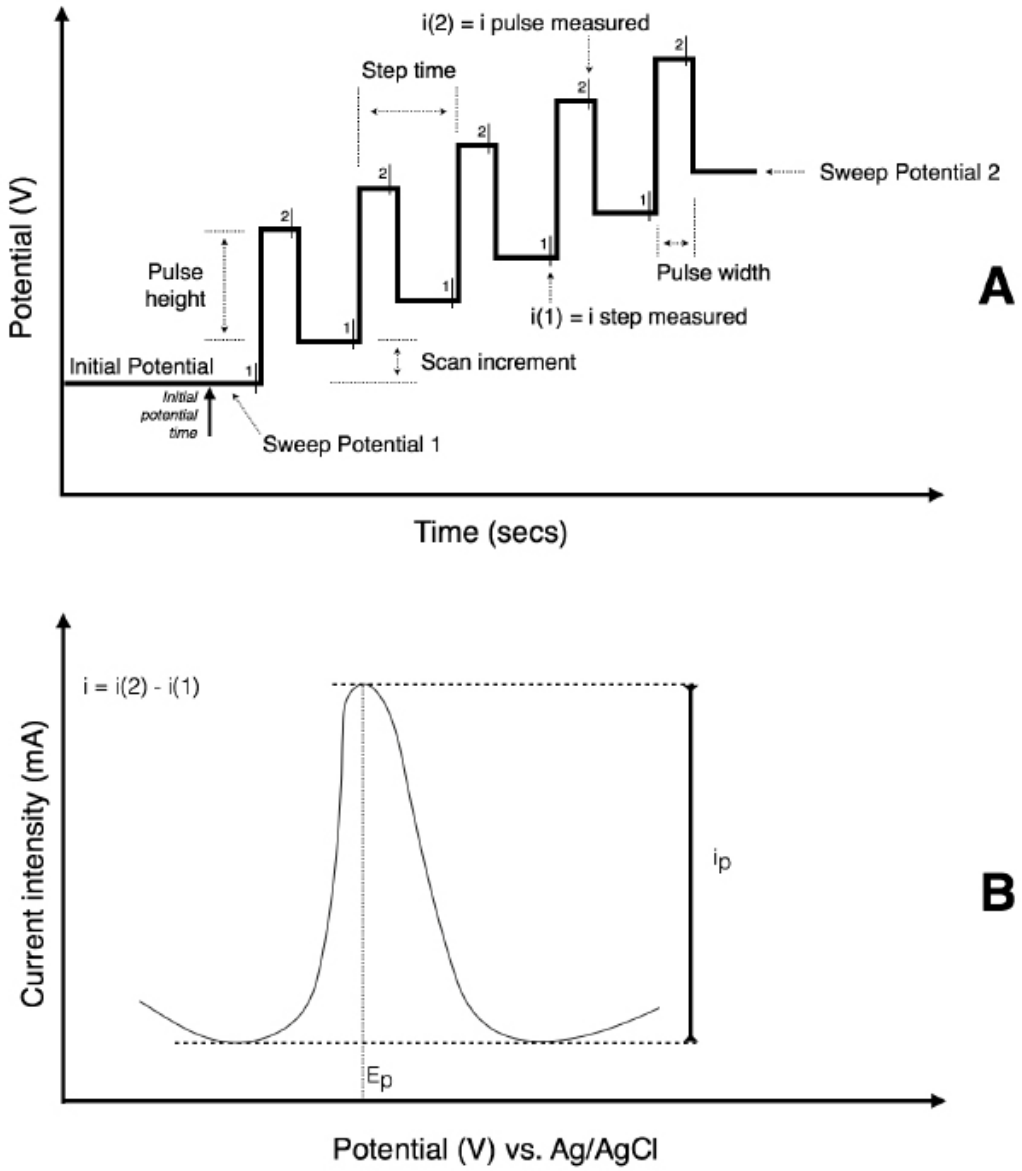

Fig. (3). DPV analysis: scheme of the potential parameters to be set (**A**) and typical voltammogram (**B**).

The voltammogram represents the difference between the two currents obtained on two applied potentials. In each pulse it goes through a maximum, showing a peak shape, being the peak's position on the potential (E_p) axis dependent of the type of analyte under study, and its height (i_p) on its concentration [20].

2.4. Square Wave Voltammetry

The SWV is another pulse voltammetry technique, which uses a potential waveform enabling to obtain more defined peaks, a good discrimination against background currents, larger dynamic concentration range and more sensitive detection of analytes than CV [11, 12]. Moreover, SWV is a faster (due to the use of frequencies between 1 and 100 Hz) technique compared with DPV [11, 12, 21], which reduces the consumption of electroactive compounds, leading to lower adsorption of species at the electrode surface (lower blocking of the electrode surface), turning out a more sensitive technique. The great advantage of pulse techniques in relation to CV is the greater capacity to discriminate the influence of capacitive current, resulting in a wider dynamic range and higher sensitivity. This technique is usually used in combination with CV because the latter provides information about the reversibility of the anodic waves.

With SWV technique, the current is measured in the WE, while between the WE and the RE, the current is swept by a symmetrical square wave, with ΔEp of amplitude, superimposed on a voltage ramp with a staircase-shaped. The current is sampled at the forward pulse (i_{fwd}) and at the end of the reverse pulse (i_{rev}) in each square-wave cycle. Measuring the current in both directions (positive towards oxidation and negative to reduction generating a peak for each of the processes) allows obtaining information concerning the oxidation or reduction of the electroactive species at the electrode surface. This dual measuring minimizes the capacitive current contribution on the total current reading. The difference between these two currents (response wave $\Delta i = i_{fwd} - i_{rev}$) is plotted *versus* the sweep potential obtaining a peak-shaped voltammogram display [11, 12]. Fig. (**4**) shows the potential parameters that are defined for the SWV analysis and the typical voltammograms obtained from the analysis: i_{fwd} curve, i_{rev} curve and Δi.

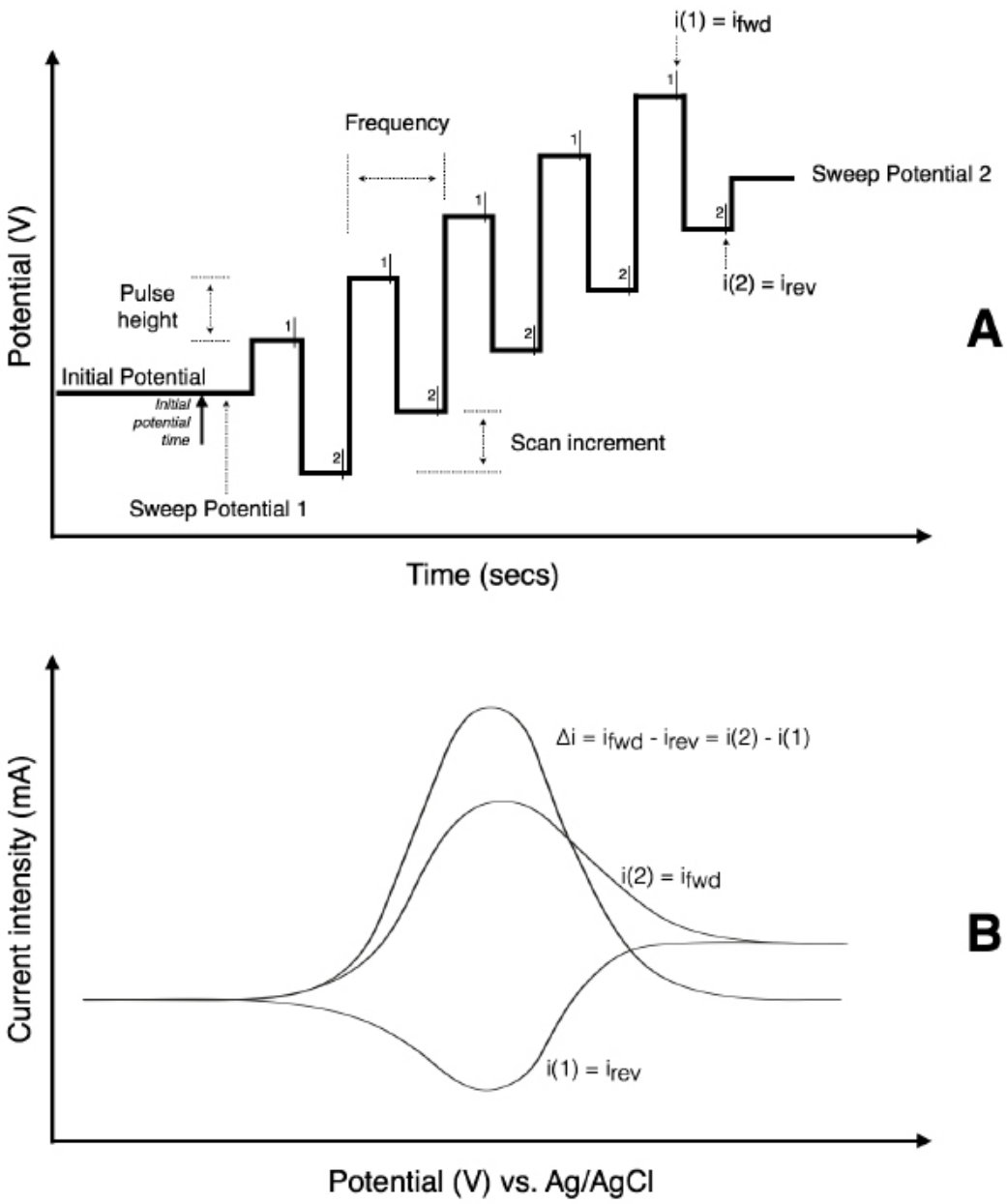

Fig. (4). SWV analysis: scheme of the potential parameters to be set (**A**) and typical voltammogram (**B**).

As DPV technique, the SWV produces faradaic response current peaks (the Δi follows Faraday's law), being the peak height directly proportional to the concentrations of electroactive compounds [10, 11]. Also, these two pulse techniques can be referred as polarography when using a dropping mercury electrode or a static mercury drop electrode as the WE [10, 11].

3. ANTIOXIDANT CAPACITY ASSESSEMENT USING VOLTAMMETRIC DEVICES: HONEY AND PROPOLIS ANALYZES

The availability of analytical tools for antioxidant screening and total phenolic compounds quantification is of major importance. The antioxidant activity of polyphenols can be evaluated according to their reactivity as a hydrogen- or electron-donating agent; capability to stabilize and delocalize the unpaired electron of radicals; reactivity with other antioxidants; and, transition metal-chelating potential [22]. So, different methods can be used for antioxidant evaluation namely, chromatography, spectrophotometry and electroanalytical techniques.

As previously mentioned, this work is focused in the use of electrochemical methodologies, not coupled to any other analytical method, for evaluating the antioxidant capacity or the quality of bee-hive products. In the first case, the studies reported in the literature only deal with antioxidant capacity assessment of propolis and honey, which could be due to the fact that, accurate and quantitative methods for antioxidant capacity measurement using electrochemical methodologies are still an object of study because of the diversity of samples and related overall mixture properties [23 - 25]. Regarding the direct use of electrochemical approaches for bee-hive products quality evaluation, only one work could be found for honey [26], where it was referred the application of differential pulse polarography (voltammetry using a mercury drop electrode) to quantify hydroxymethylfurfural and fructose levels. This is unexpected since these techniques do not require a time-consuming sample treatment and are cost-effective methods enabling fast, simple and sensitive analysis of bioactive compounds associated with radicals scavenging and so, to the antioxidant capacity [20, 27].

In the literature [28 - 34], the use of electrochemical devices for bee-hive products analysis is mainly focused in antioxidant capacity assessment (Table **1**). For other matrices, like plants, fruits, beverages or algae, voltammetric methods and electrochemical biosensors have been described for quantifying single or total phenolic compounds as well as to assess the antioxidant capacity in standard solutions or in extracts [15, 35 - 44]. Indeed, voltammetric methods (*e.g.*, CV, DPV or SWV) have been applied as a fast screening tool for identifying novel antioxidants, being an attractive approach although they have not yet found a widespread use [14]. The capability of electrochemical devices to assess antioxidant capacity relies in the fact that phenolic compounds are prone to redox inter-conversion. Indeed, most of the phenolic compounds are electrochemically active at moderate oxidation potentials and so, the use of electrochemical methods may be preferable compared to spectrophotometric, chromatographic, capillary electrophoretic or chemiluminescent techniques, since they are less prone to interferences from non-electroactive substances [40]. Also, due to the different mechanisms of antioxidant action of some phenolic compounds (*e.g.*, flavonoids), electroanalytical methods are viewed as the most suitable techniques for antioxidant capacity evaluation and for electroactive species characterization [45].

Table 1. Electrochemical studies in bee-hive products and their isolated compounds.

Electrochemical technique	Sample	Type and conditions of analysis	Instrumental conditions	Ref.
Direct current polarography	Honey of different floral sources and main constituents (flavonoids, phenolic acids, amino and organic acids, and carbohydrates).	Hydrogen peroxide scavenging (HPS) activity of honey and isolated compounds. Analysis with Clark-Lubs borate buffer (pH 9.8) using H_2O_2 concentrations higher than $1x10^{-3}$ M.	Three-electrode system with a dropping mercury working electrode, a saturated calomel reference electrode and Pt-foil as auxiliary electrode. Mercury dropping time of 1 s and the current-potential curves recorded at room temperature using starting potential at 0.10 V and scan rate of 10 mV/s. Calibration curve with decrease of the anodic current peak of H_2O_2 in presence of honey samples (signal) *vs*. mass of added samples. The slope used was the measure of HPS activity.	[28]

(Table 1) contd.....

Electrochemical technique	Sample	Type and conditions of analysis	Instrumental conditions	Ref.
Direct current polarography	Propolis	Hydrogen peroxide scavenging (HPS) activity of propolis using Clark-Lubs Borate buffer with pH 9.8 and H_2O_2 concentrations higher than $1x10^{-3}$ M.	Three-electrode cell with a dropping mercury WE, a saturated calomel RE and Pt foil as AU. Mercury dropping time of 1 s and the current-potential curves recorded at room temperature using starting potential at 0.10 V and scan rate of 10 mV/s. Calibration curve with decrease of the anodic current peak of H_2O_2 in presence of propolis samples (signal) *vs*. mass of added samples. The slope used was the measure of HPS activity.	[29]
Cyclic voltammetry	Flavonoids and caffeic acid esters isolated from propolis	Redox properties of isolated compounds from propolis using acetonitrile under Argon atmosphere	Electrochemical cell of three electrodes containing a working and auxiliary Pt and reference saturated calomel electrode. Voltammograms were recorded between -3 and +3 V.	[30]
Cyclic voltammetry	Chrysin isolated from propolis	Redox properties of an isolated compound extracted from propolis and dissolved in ethanol was analyzed in a series of Britton-Robinson buffer solutions from pH 2.0 to 9.0.	A conventional three-electrode system with a saturated calomel RE, and Pt wire counter electrode and static mercury drop working electrode. Analysis were carried out in the potential interval of -1.8 to -1.2 V.	[31]
Cyclic voltammetry	Propolis	Antioxidant capacity of methanolic extracts of propolis in pH 7 phosphate buffer solution. Ascorbic and gallic acids used as standards in the calculation of antioxidant capacity.	Electrochemical cell with glassy carbon working electrode, a Pt wire CE, and a saturated calomel RE. The potential was swept in inverse scanning mode starting from -0.2 to +0.8 V with a scanning rate of 100 mV/s. Antioxidant capacity calibration curve using the area below the anodic curve of the voltammogram, as signal, *vs* standard's concentrations.	[32]

(Table 1) contd.....

Electrochemical technique	Sample	Type and conditions of analysis	Instrumental conditions	Ref.
Cyclic voltammetry	Propolis and pinocembrin and galangin isolated from propolis	Antioxidant activity of propolis extracts and isolated compounds. Analysis using hydro-methanolic solutions with pH=6.6 phosphate buffer. Ascorbic acid used as standard compound for antioxidant capacity comparison.	A three-electrode system applied with an Ag/AgCl RE, a Pt WE and a Pt wire CE. Voltammograms were recorded from -0.1 to +1.3 V with a scan rate of 100 mV/s. Total charge (peak area) below the anodic wave curve of the voltammogram was used *vs* standard's concentrations for curve fitting method.	[33]
Cyclic voltammetry	Propolis	Antioxidant capacity of methanolic propolis extract in pH=7 phosphate buffer aqueous solution. Ascorbic and gallic acids used as standards for evaluating antioxidant capacity.	A three-electrode cell was used with a glassy carbon WE, a Pt-wire CE and a saturated calomel RE. The potential sweep was in the interval -0.2 to 0.8 V with a scanning rate of 0.1 mV/s. Calculations using anodic area *vs*standard's concentrations.	[34]

However, the presence of two or more species in a sample, with similar redox properties, may be a major problem, namely when CV is applied, which can be partially overcome by applying high resolution pulse voltammetric methods like DPV and SWV that are able to enhance the discrimination between the target and the interferent molecules [46]. Nevertheless, in some situations, it is reported that, contrary to what should be expectable, CV may have an apparent higher sensitivity compared to DPV technique, providing information on the reversibility of the anodic waves [41]. So, the simultaneous use of different voltammetric methodologies can be a clear advantage [14]. Even so, the potential user of these electroanalytical methods must be aware of some possible limitations, namely the occurrence of overlapping signals for multi-component mixtures analysis, especially if one compound is present in excess and the electrode sensitivity is reduced due to the sluggish electron transfer kinetics or fouling of the electrode, through contamination or passivation of the electrode surface [14, 42, 46]. The latter leads to the formation of insulating films at the electrode surface due to adsorption effects, which may cause to a non detectable anodic wave potential signal [14, 42, 46].

Finally, it should be kept in mind that these electroanalytical methods, although

being inexpensive, simple, rapid, sensitivity and portable, should be used mostly for basic analyses since the selective determination of a particular compound is, usually, not feasible, being in general not possible to establish which compound is responsible for each peak observed, and so only some reasonable assumptions might be tentatively highlighted when analyzing real samples [42, 44]. Nevertheless, voltammetric methods, used individually or in combination, are attractive screening techniques for raw samples enabling the identification of potential antioxidants with a greater confidence compared to well-known chemical assays (*e.g.*, DPPH or the ferrous chelation methods) [14]. In addition, electrochemical techniques (*e.g.*, CV and SWV) may even be used to guide the isolation of antioxidant natural products from complex crude samples (*e.g.*, marine algae extracts) contributing to the isolation of antioxidant molecules [47].

Recently, the CV technique was used to determine the antioxidant capacity of flavonoids-metal ions complexes as well to discuss flavonoids/metal ions interactions [48]. Also, electrochemical ultra-micro sensors were developed and satisfactorily applied in the determination of synthetic and natural antioxidants in oils, based on SWV data [49]. So, electrochemical approaches (*e.g.*, CV and SWV) may provide new insights of the process and kinetic related to the electrochemical oxidation of phenolic compounds, namely flavonoids, contributing to a deep knowledge of physical and chemical properties of antioxidants as well as for understanding the mechanisms of their oxidation or reduction processes [50]. Also, several works using DNA- or purine-based electrochemical biosensors have been applied to evaluate antioxidant capacities of different matrices (*e.g.*, plant extracts, beverages) [42, 51 - 56]. Nanomaterials, such as metal nanoparticles (MNPs) and quantum dots (QDs), applications for *in vitro* antioxidant capacity assessment in complex samples were recently reviewed and discussed [57]. So, electrochemical techniques, alone or combined to traditional analytical methods (*e.g.*, chromatography), can be a practical tool for evaluating the antioxidant of different matrices.

However, the direct application of electrochemical methods for bee-hives products antioxidant capacity assessment has been rarely reported in the literature. The studies used voltammetric or polarographic assays, and were focused mainly in the analysis of propolis [29, 30, 32 - 34, 58, 59] and less frequently of honey

[28]. Phenolic compounds isolated from propolis were also electrochemically characterized by CV, and in particular the antioxidant activity of chrysin, a widely distributed flavonoid in propolis, was evaluated by linear sweep voltammetry (LSV) on a static mercury electrode [31]. Unfortunately, no work has been found by the authors regarding the direct application of an electrochemical device for bioactivity capacity assessment of pollen.

In the next section, the application of electrochemical devices to assess antioxidant capacity of bee-hive products is reviewed, namely for honey and propolis. Concerning honey, CV assays of our research group are also presented and discussed.

3.1. Honey's Antioxidant Capacity Evaluation using Voltammetric Sensors

In the literature, it was only possible to find one work that describes the application of a polarographic method for assessing antioxidant activity of honeys. Gorjanović *et al.* [28] proposed a direct current polarographic assay to evaluate HPS activity of honey from different floral sources and its main constituents (*e.g.*, flavonoids, phenolic acids, amino acids, organic acids and carbohydrates). As for propolis extracts analysis [29], the assay was based on the decrease of anodic current of hydrogen peroxide complex, formed in alkaline solution, at the potential of mercury dissolution. Antioxidant activity of honey reflected an integrated action of different constituents, both phenolics and non-phenolics [28]. Also, the potential of polarography for antioxidant capacity assessment, already demonstrated for propolis extracts, was confirmed for honey analysis [28, 29].

To further investigate the potential applicability of a voltammetric approach to evaluate redox activity of honey, CV preliminary assays were conducted by our research team in Portuguese honeys. The pollinic profiles (based on the melissopalynology analysis), color classification (according to the quantitative mm Pfund scale based on spectrophotometry assays) and the potentiometric behavior (recorded using an electronic tongue) were previously reported by the research group [60]. Honeys were classified as monofloral, according to their floral origin (*e.g.*, *Castanea* sp., *Echium* sp., *Erica* sp., *Lavandula* sp., *Prunus* sp.

and *Rubus* sp.) and their color range included white (17 < mm Pfund ≤ 34), extra light amber (34 < mm Pfund ≤ 50), light amber (50 < mm Pfund ≤ 85), amber (85 < mm Pfund ≤ 114) and dark amber (> 114mm Pfund). Honey samples were dissolved in water (5 g of honey with 50 g of water) before being electrochemically analyzed. The voltammetric equipment consisted of a potentiostat-Galvanostat device (PG580, Uniscan) with a typical 3-electrodes system, using the American current polarity convention. A silver electrode (M295Ag, Radiometer) was used as the WE, a Pt electrode (M241Pt, Radiometer) used as the CE and an Ag/AgCl electrode (M90-02, Orion) as the RE (Fig. **5**).

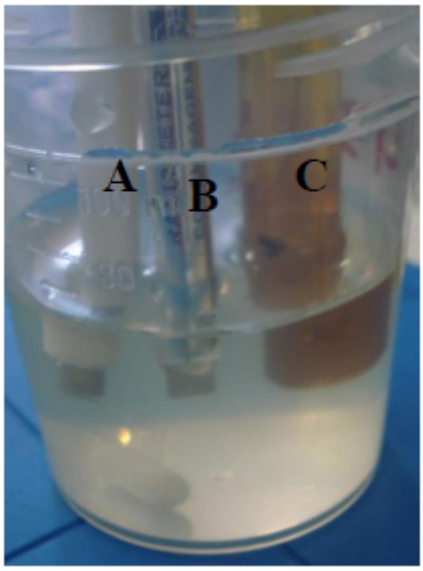

Fig. (5). The 3-electrodes system used: **A**) silver (M295Ag, Radiometer) working electrode; **B**) Pt (M241Pt, Radiometer) counter electrode; **C**) Ag/AgCl (M90-02, Orion) reference electrode.

An example of the cyclic voltammograms recorded during the aqueous honey solution analysis is shown in Fig. (**6**). As can be seen, only one anodic wave curve was obtained, showing a typical irreversible oxidation electrochemical process with one anodic peak at a negative potential (≈ -250 mV). Similar electrochemical profiles (data not shown) were observed for all monofloral honeys analyzed, although with slight different oxidation potentials, varying from -250 up to -100 mV, depending on the color and floral origin of the honey sample. The wide

anodic curve may be attributed to the combined response of several electro-active chemicals present in the honey samples (*e.g.* flavonoids, phenolic acids and water soluble vitamins) that had different oxidation potentials [33].

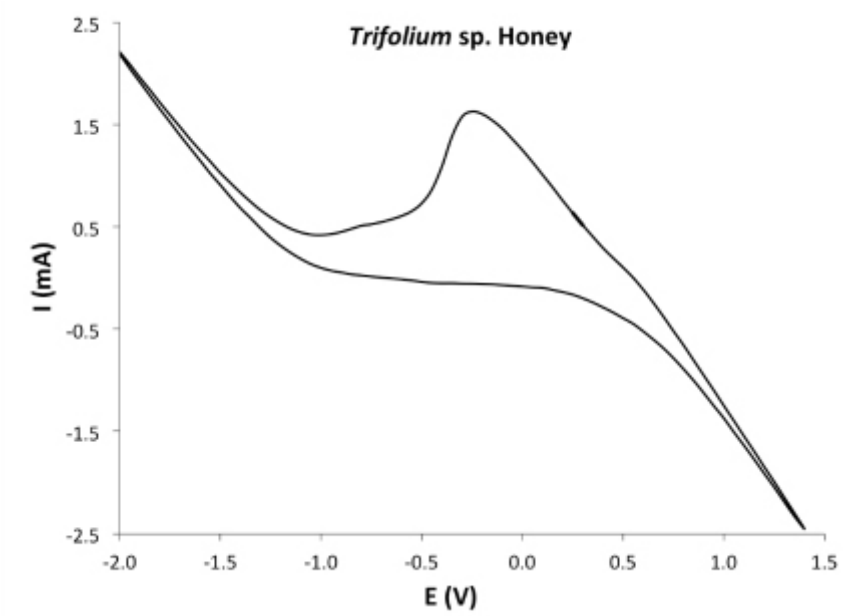

Fig. (6). A typical cyclic voltammograms recorded of an aqueous honey solution (*e.g. Trifolium* sp. monofloral honey).

Fig. (**7**) exemplifies the cyclic voltammograms recorded for monofloral honeys of *Lavandula* sp. with different colors (varying from white to dark amber). The similarity observed in the oxidation potentials and global voltammetric profiles may indicate that Portuguese *Lavandula* sp. monofloral honeys have analogous chemical composition regarding electro-active species, regardless honey color. However, the anodic peak current and the anodic curve area increased with the increasing darkness of the *Lavandula* sp. honey (from white to dark amber, Fig. (**7**), which could be related to the known higher content of phenolic compounds (and by consequence of the related antioxidant capacity) of dark colored honeys compared to light colored honeys [61 - 65]. Indeed, for the honey samples shown in Fig. (**7**), a positive linear correlation could be established between the mm Pfund values of the *Lavandula* sp. honeys and the respective anodic peak current

intensities (*R*-Pearson = 0.968), confirming the previous conclusion (*i.e.*, > peak current → > mm Pfund).

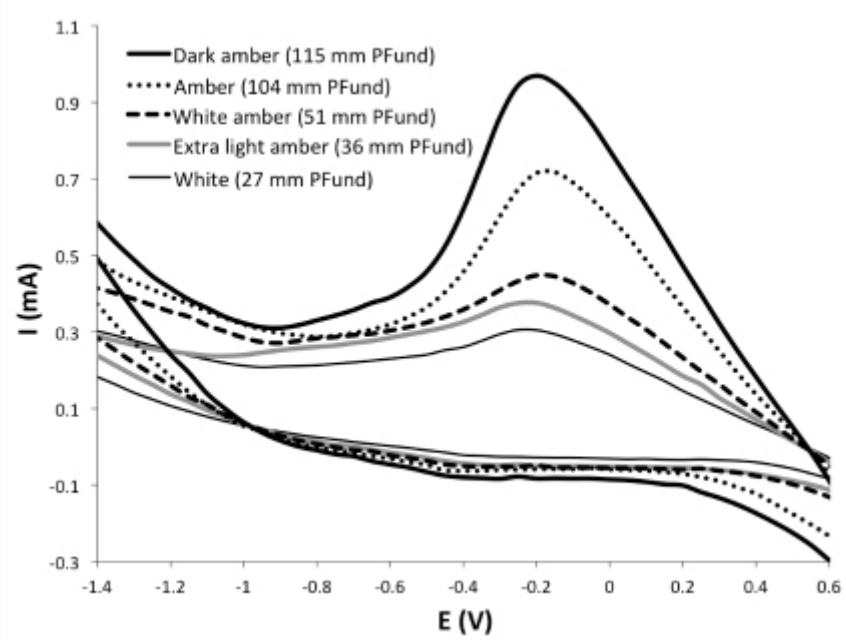

Fig. (7). Cyclic voltammograms recorded for monofloral honeys of *Lavandula* sp. with different colors (varying from white to dark amber).

Similar behaviors could be found for the others Portuguese monofloral honeys (data not shown). Moreover, similar voltammetric behaviors were observed for honey samples with different floral origins but with the same color classification (based on the mm Pfund scale), although with anodic peak current at slight different potentials and with different anodic curves areas. This fact may indicate that the Portuguese monofloral honeys with the same color may contain similar electro-active species although in different levels, and so, with different expected antioxidant capacities. As an example, Fig. (**8**) depicts the voltammetric profiles of dark amber honeys with different floral origins. From the figure it may be inferred that both the anodic peak current and the anodic curve area increase in the order *Castanea* sp. < *Echium* sp. < *Rubus* sp. < *Lavandula* sp. < *Prunus* sp. < *Erica* sp. < *Trifolium* sp. honeys, which may indicate that both the phenolic content and the antioxidant capacity of Portuguese monofloral honeys within the

same color range may vary. Indeed, it has been reported that extracts with lower oxidation potential values may exhibit higher antioxidant capacity [66].

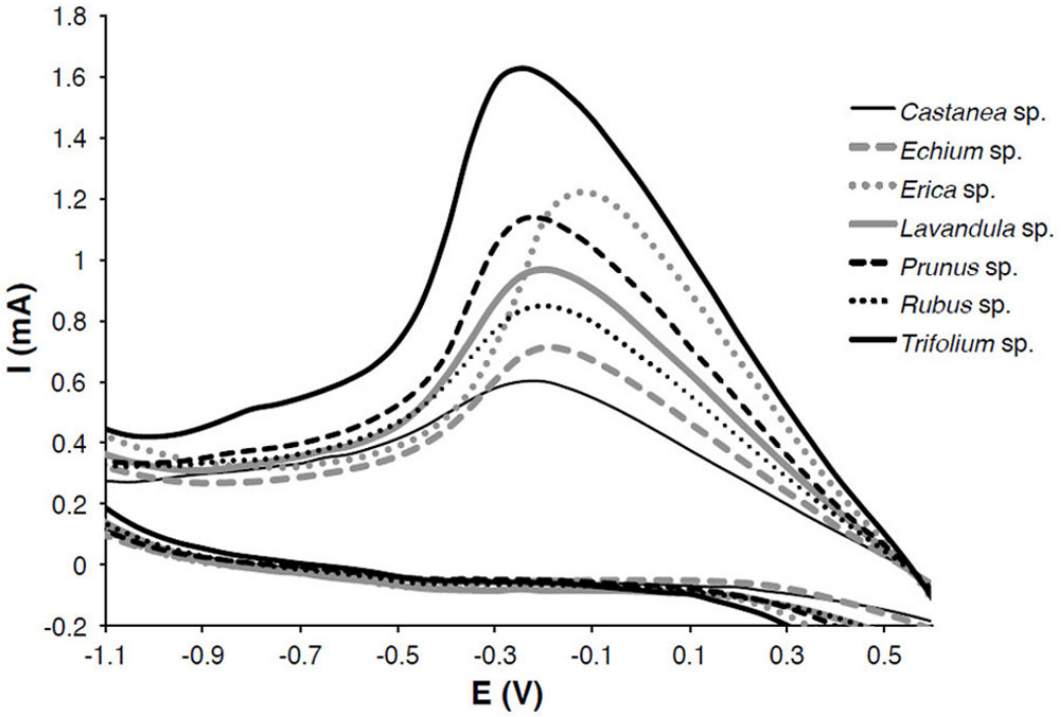

Fig. (8). Voltammetric profiles of dark amber honeys with different floral origins.

3.2. Propolis' Antioxidant Capacity Evaluation using Voltammetric Sensors

Several works report the capability of direct voltammetric methods (including CV, DPV and SWV) and polarographic approaches to assess the antioxidant activity of propolis extracts, demonstrating the possibility of applying these electrochemical techniques on resinous substances. Also, the quality of the results obtained with these electroanalytical tools demonstrate that these methodologies could be recommended as appropriate for determination of antioxidant capacity of propolis extracts being a possible, fast and cost-effective alternative to widely accepted assays.

One of the first works reported in the literature dates back to 1995 [30] and describes the evaluation of the redox properties of flavonoids isolated from propolis, by CV in acetonitrile extracts. The aprotic solvent reduced the radical

intermediates reactivity, enabling the identification of the redox steps and intermediates compounds. The work of Rapta *et al.* [30] demonstrated the existence of a negative correlation between lipid antioxidant properties of flavonoids and caffeic acid esters isolated from propolis and their oxidation potential. After, Zheng *et al.*[31] studied the electrochemical behavior of chrysin, a flavonoid isolated from propolis, by LSV and CV. The assays were conducted using an electrochemistry workstation and a conventional three-electrode system (a standard saturated calomel as the RE, a platinum (Pt) wire as the CE and a static mercury drop as the WE). The approach allowed to propose an electrochemical reduction mechanism of chrysin [31]. Also, the ability of chrysin for scavenging active oxygen radicals yielded by the autoxidation of pyrogallol was evaluated showing the good antioxidant capacity of this flavonoid. More recently, Laskar *et al.* [33] studied by CV the *in vitro* antioxidant capacity of aqueous and ethanol extracts of propolis from India. A potentiostat–galvanostat apparatus was used coupled to a three-electrode system, including an Ag/AgCl as the RE, a platinum (Pt) electrode as a WE and a Pt wire as a CE. Each extract was diluted using the same volume of 0.2 M phosphate buffer (pH 6.6). Voltammograms were recorded from -100 to +1300 mV with a scan rate of 100 mV s^{-1}. Both type of extracts showed an irreversible electrochemical behavior with one anodic peak at oxidation potentials less positive than that recorded for ascorbic acid standard solutions. Furthermore, Falcão *et al.* [58] characterized Portuguese propolis based on its electrochemical behavior. The voltammetric evaluation was performed by CV, DPV and SWV, in ethanolic extracts (-0.5 to +200 mV). A potentiostat with a typical three-electrode cell (Ag/AgCl as the RE, a Pt wire as CE and a glassy carbon disk as the WE) was used. The redox profiles of propolis phenolic extracts were studied by CV, while DPV and SWV enabling the quantification of electroactive species present in the different extracts of propolis. Irreversible oxidation processes were observed at different potentials depending on the geographical origin of the samples allowing the discrimination of propolis samples by geographical origin. Rebiai *et al.* [34] evaluated the antioxidant capacity of methanolic Algerian propolis extracts by CV (-200 to +800 mV, at a scan rate of 100 mV/s). A potentiostat device connected to a typical 3-electrodes system was used (a glassy carbon electrode as the WE, a Pt wire as the CE and a Hg/Hg_2Cl_2 as the RE). The CV results showed that propolis

extracts had electrochemical behaviors slight different from those recorded for standard phenolic solutions, suggesting a different electroactive chemical composition, although possessing antioxidant capacity under *in vitro* conditions. Lourenço *et al.* [59] also applied CV to assess the antioxidant capacity of propolis ethanol extracts from Azores (Portugal). The preliminary study showed that the extracts possessing higher antioxidant activities also had higher antibacterial activities. More recently, Rebiai *et al.* [32] tested polyphenols extracted from Algerian propolis, using CV in aqueous media. Also, antioxidant capacities of propolis methanol extracts were evaluated by CV. This electrochemical technique provided a qualitative composition of each extract as well as an estimative of the total polyphenols content in each extract. The propolis methanol extracts presented typical irreversible oxidation processes similarly to that recorded for standard solutions, although with oxidation potentials more positive than ascorbic acid and lower than gallic acid. Under the electrochemical conditions used, the CV data did not indicate that propolis extracts had an antioxidant capacity lower than gallic acid and greater than ascorbic acid-Indeed, propolis extracts showed a higher antioxidant capacity compared with that of gallic acid standard solution, contrary to the expected behavior.

One work reported the application of direct current (DC) polarography for assessing the antioxidant capacity of commercial propolis extracts available in Serbia [29]. In this study, the antioxidant activity was evaluated by plotting of the polarographic anodic current decrease of an initial alkaline solution of H_2O_2 due to the gradually addition of pre-established volumes of propolis ethanol extracts. A Polarographic Analyzer PAR was used for the electrochemical measurements, with a conventional three-electrode cell (a dropping mercury electrode was used as the WE, a saturated calomel electrode as the RE and the Pt foil as the CE).

CONCLUSION

The examples presented on electrochemical techniques application in the evaluation of antioxidant capacity or determination of polyphenolic compounds in food, beverages and related plant extracts showed the added value of these techniques. They provide supplementary insight about redox-processes. These techniques have been considered as complementary or alternative in the

evaluation of antioxidant power, mainly due to the speed of analysis with a reduced sample treatment. The number of studies shows that the application of these methodologies in the study of antioxidant properties in honey and propolis samples is in an early stage. Considering the economic, nutritional and human health importance of these products, it is expected that the electrochemical studies become an essential analytical tool for the characterization of these samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding this publication.

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