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Redox Behavior of the Ellagitannin Oenothein B and Ellagic Acid at a Glassy Carbon Electrode

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Oenothein B and ellagic acid are polyphenolic compounds, and their oxidation mechanism is pH-dependent, in one step, involving a different number of electrons and protons. Oenothein B and ellagic acid first oxidation occurs at a lower potential, corresponding to the quasi-reversible oxidation of the phenolic hydroxyl groups. The oxidation of both compounds leads to the formation of electroactive products that undergoes an irreversible oxidation process at higher potentials. The electrochemical characterization of their redox behavior brought useful data about their chemical stability, antioxidant and pro-oxidant activity, enabling a comprehensive understanding of their redox mechanism.

Keywords: Oenothein B, Ellagic acid, Antioxidant properties, Glassy carbon electrode.

1. INTRODUCTION

Tannins are polyphenols which occur commonly in plants used as foods and beverages, and they can be subdivided into hydrolysable and condensed tannins [1]. A group of hydrolysable tannins, called ellagitannins, is defined as hexahydroxydiphoyl esters of glucose, which liberates ellagic acid on acid hydrolysis [2]. Such class of natural products has a wide array of pharmacological properties including anti-inflammatory, antibacterial and antitumor activities [2,3]. Moreover, ellagitannins showed remarkable antioxidant and free radical scavenging actions, which might be involved in the mechanisms of their biological activities [4,5].

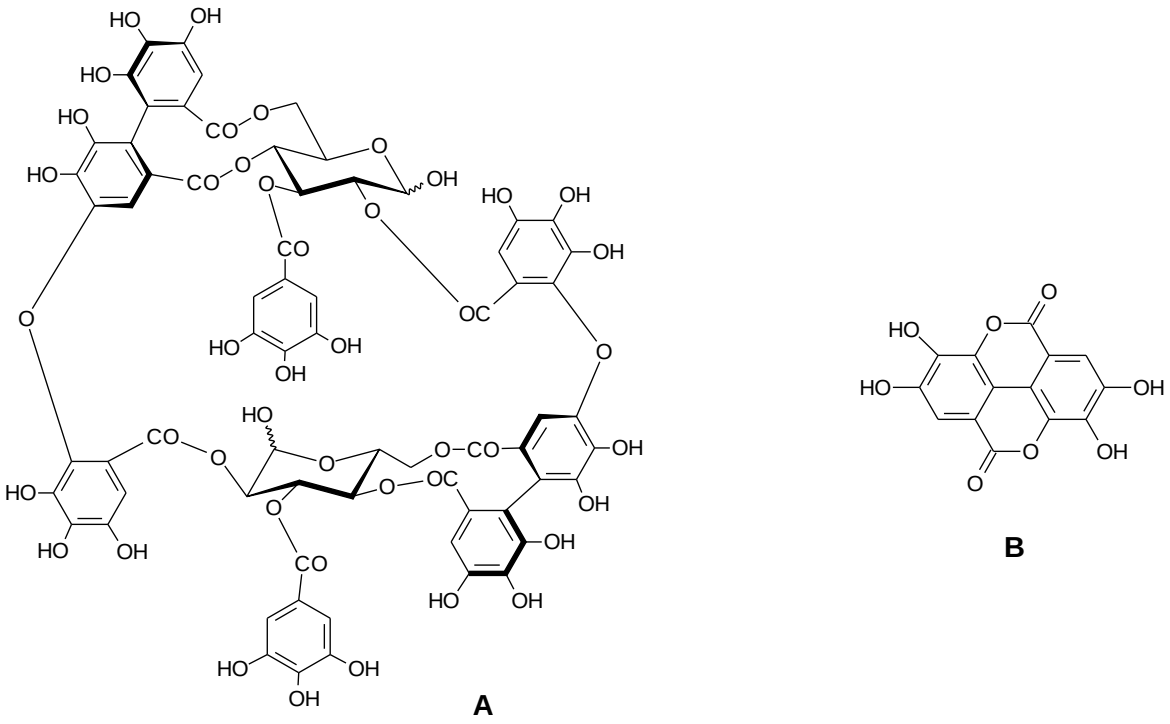

Figure 1. Chemical structures of oenothein B (A) and ellagic acid (B)

Oenothein B (Figure 1) is a dimeric macrocyclic ellagitannin first isolated from leaves of *Oenothera erythrosepala* and later from several medicinal plants belonging to Onagraceae, Lythraceae and Myrtaceae families [6]. The presence of twenty two phenolic hydroxyl groups in the structure contributes to its high antioxidant activity as scavenger of $O_2^{\cdot-}$ and DPPH radicals, with IC_{50} of 0.9 and 6.1 μM, respectively [7,8]. In addition, oenothein B has exhibited antiviral [9], antibacterial [10], anti-inflammatory [7], antimutagenic [11] and antitumor [12-16] activities. The anti-inflammatory effect is related to the inhibition of lipoxygenase and hyaluronidase activities and the obstruction of myeloperoxidase's release [7], while the antitumor activity may be due to the improvement of the host-immune system via induction of IL-1β [16].

Ellagic acid (Figure 1) naturally occurs in various fruits and nuts, such as raspberries, strawberries, walnuts, mango, kernel and pomegranate [17], usually as glycosides or linked in the ellagitannin structures [2], and only small quantities exist as free acid in plants [17]. Several biological activities such as antibacterial, antiviral, antifibrotic, anti-inflammatory and anticancer have been described for this compound [18]. Moreover, various studies using a wide range of antioxidant assays have demonstrated the strong free radical scavenging activity for this acid [19, 20]. Recently, voltammetric techniques were applied for ellagic acid determination in plant materials and to investigate its electro-oxidation mechanism [17, 21-27].

Since the electroanalysis can offer useful data about electro-oxidation mechanisms, this study may improve the understanding of the redox behavior of oenothein B and its metabolite, ellagic acid, leading to predictions of their antioxidant and/or pro-oxidant properties, as well as chemical stabilities [21-27].

Therefore, this work aimed to compare the electrochemical behavior of oenothein B and ellagic acid on a glassy carbon electrode in different electrolyte media using cyclic, differential pulse and square-wave voltametry (CV, DPV and SWV, respectively).

2. MATERIAL AND METHODS

2.1 Materials and Reagents

Oenothein B was isolated from *E. uniflora* leaves (deposit number UFG 25477). Dried and grounded leaves (1.0 kg) were extracted with 50% acetone, at room temperature. The acetone was removed under reduced pressure and the suspended aqueous extract was filtered to eliminate fats and chlorophylls. Following, a liquid-liquid extraction with ethyl acetate (10 x 150 mL) was carried out. The aqueous layer was freeze-dried to yield a 122 g extract, which was dissolved in methanol (800 mL) to separate soluble (87 g) and insoluble (33 g) methanolic extracts. Six portions of the soluble methanolic fraction were submitted to column chromatography over Diaion HP-20 (200 g) and eluted with a decreasing polarity gradient of H_2O/MeOH. The 80% MeOH eluate (13 g) was chromatographed over Sephadex LH-20 (200 g), eluted with the same gradient of water and aq. MeOH to afford oenothein B (0.5 g) [28].

Ellagic acid was purchased from Sigma (Sigma–Aldrich, Sternheim, Germany). A stock solution was prepared in methanol and stored at +4 °C. Solutions of different concentrations were prepared by dilution of the appropriate quantity in supporting electrolyte. All supporting electrolyte solutions, were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 μScm -1).

2.2 Apparatus

Voltammetric experiments were carried out using a μAutolab running with GPES 4.9 software, Eco-Chemie, Utrecht, The Netherlands. Measurements were carried out using a three-electrode system in a 1 mL one-compartment electrochemical cell (Cypress System Inc., USA). Glassy carbon electrode (GCE, d =1.0 mm) was the working electrode, Pt wire the counter electrode and the Ag/AgCl (3 $molL^{-1}$ KCl) reference electrode. The GCE was polished using alumin particles of 2 μm (Arotec, BR) before each electrochemical experiment.

After polishing, it was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer supporting electrolyte and voltammograms were recorded until a steady state baseline voltammograms were obtained. This procedure ensured very reproducible experimental results. The experimental conditions for differential pulse (DP) voltammetry were: pulse

amplitude 50 mV, pulse width 50 ms and scan rate 10 mVs^{-1}. For square wave (SW) voltammetry were: pulse 60 mV, frequency 30 Hz and potential increment 4 mV, corresponding to an effective scan rate of 121 mVs^{-1}.

The pH measurements were carried out with a Quimis pH-meter with an Quimis combined glass electrode. All experiments were done at room temperature (25 ± 2 ^{o}C) and microvolumes were measured using Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA).

2.3 Acquisition and Presentation of Voltammetric Data

The DP voltammograms presented were background-subtracted and baseline-corrected using the moving average application with a step window of 2 mV included in GPES version 4.9 software. This mathematical treatment improves the visualisation and identification of peaks over the baseline without introducing any artefact, although the peak intensity is, in some cases, reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented in all plots were determined from the original untreated voltammograms after subtraction of the baseline.

3. RESULTS AND DISCUSSION

3.1 Cyclic Voltammetry

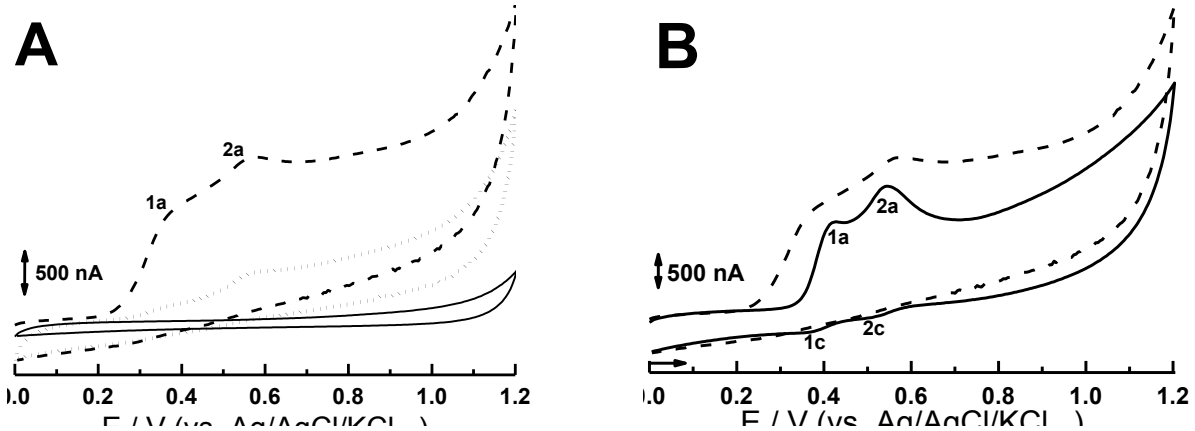

Figure 2. First (- - -) and third (●●●) cyclic voltammograms obtained at GC electrode in a 0.1 M PBS, pH 5.0, containing 40 mM oenothein B **(A)** and 80 mM of ellagic acid **(B)**. Solid curve: blank recorded in PBS. ν = 100 mV s^{-1}.

The cyclic voltammograms recorded in a 0.1 M phosphate buffer, pH 5.0, containing 40 mM oenithein B solution are presented on Figure 2A. Two well defined anodic peaks, peak 1a and 2a were detected at + 0.36 V (E_{p1a}) and + 0.55 V (E_{p2a}), but high decrease in the anodic current peaks were observed in the second cycle. Reversing the scan direction, no or very discrete cathodic peaks were observed. When oenothein B was substituted by ellagic acid, the behavior was a little different: the anodic current peaks also decreased at the second cycle, but two low current intensity cathodic

shoulders were detected at + 0.42 V (E_{p1a}) and + 0.54 V (E_{p2a}), which better defined in the second scan, Figure 2B.

The redox pair E_{p1a}/E_{p1c} observed for ellagic acid corresponds to the electrochemical oxidation of the *orto*-di-phenolic hydroxyl groups, and similar behavior should be expected also for oenothein B, since the oxidation mechanisms for both compounds are similar [17,21-24].

The effect of scan rate on the first anodic process for oenothein B and ellagic acid showed that the electrochemical processes are controlled by diffusion and adsorption, respectively. □(E_{p1a} - E_{p1c}) is < 30 mV for the ellagic acid, anoher indicative of the adsorption phenomenon on the electrode surface. In oenothein B solution, besides the complexity of the molecule, I_{p1a} (current peak at E_{p1a}) increased linearly with the square root of the scan rate.

In fact there are some reports showing that the anodic oxidation of ellagic acid was mixed controlled process (diffusion and adsorption) [17, 21] on unmodified electrode surfaces, while at a carbon nanotubes (CNT's) carbon paste - modified electrodes, the same process was controlled by diffusion, at least for low scan rates (25 $mVs^{-1} \leq \nu \leq 105$ mVs^{-1}) [22].

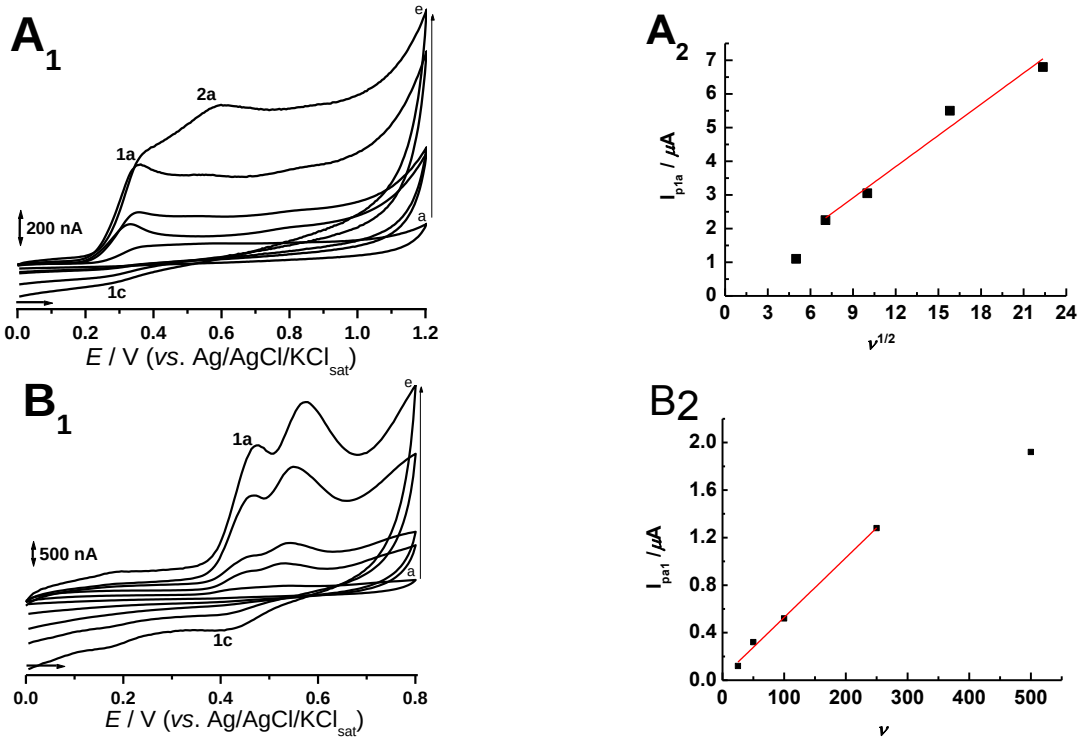

Figure 3. Cyclic voltammograms obtained for 20 mM Oenothein B (**A**) and ellagic acid (**B**) in pH 5.0 0.1 M PBS at different scan rates a → e: 25; 50; 100; 250 and 500 mV s^{-1}.

3.2 Differential Pulse Voltammetry

The electrochemical oxidation of oenothein B and ellagic acid was studied by using Differential Pulse Voltammetry in 0.1 M phosphate buffer at

pH 5.0. To evaluate the adsorption effect on the electrochemical processes of both compounds, successive voltammograms were obtained and the results are presented on Figure 4.

The first DP voltammogram obtained at 10 mM oenothein solution showed a well defined and large peak 1a, at E_{p1a}= + 0.31 V, but this peak clearly involves another electrochemical process (probably, the anodic peak 2a observed in CV assays) and therefore, the expected value of the width at half height ($W_{1/2}$ of 45 mV) corresponding to an electrochemical reaction involving two protons and two electrons, similarly to the catechol oxidation [27], was not observed, Figure 4A.

The ellagic acid, in the same experimental conditions, showed the first oxidation peak, E_{p1a}, at a more positive value, E_{p1a} = + 0.39 V (Figure 4B), which was attributed at the oxidation of catechol moiety. In both cases, the current levels decreased drastically, san by scan.

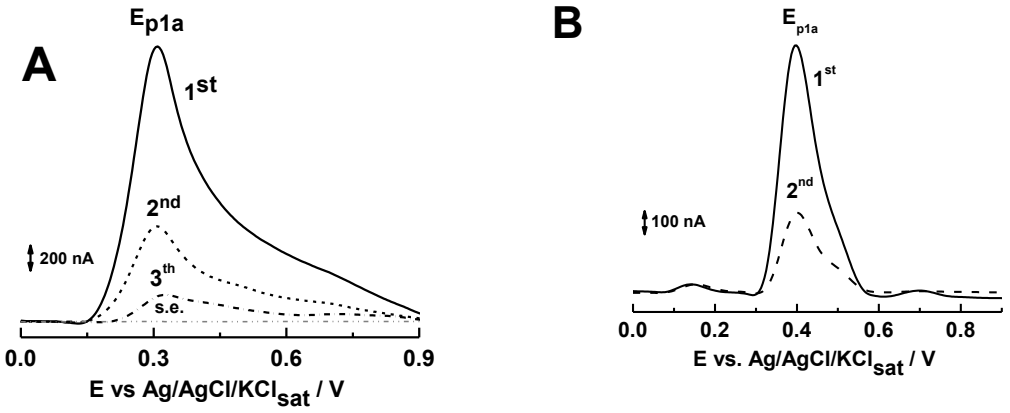

Figure 4. Successive DP (1^{st} , 2^{nd} and 3^{th}) voltammograms obtained in 0.1 M phosphate buffer solution, pH 5.0, containing: (**A**) 10 mM oenothein B and (**B**) 10 mM of ellagic acid. Pulse amplitude of 50 mV and scan rate of 10 mV s^{-1}.

The peak potential pH dependence of both compounds in different electrolytes with 0.1 M ionic strength was investigated running DPV at a clean GCE surface.

Peak 1a of enothein B shifted to less positive potentials according to: E_{p1a} / mV = 434.5 mV – 29.5 pH in the range of 2.0 ≤ pH ≤ 5.0. In comparison with the obtained using CV it can be conclude that at low scan rate, the oxidation process at E_{pa1} is an adsorption-diffusion mixed controlled. However, the same process is diffusion controlled in the range of 5.0 ≤ pH ≤ 7.0 according to: E_{p1a}/mV = 561.7 – 55 pH), indicating that same number of protons and electrons are involved in the oxidation process. At pH > 7.0, the process is pH-independent, Figure 5A2. The presence of two pH-dependent linear regions and one region pH-independent shows that there are an acid-base equilibrium involving two different species and another specie, which is electrochemically oxidized in the proton absence. The experimental pK_{a1} and pK_{a2} values for oenothein B, determined using the linear fitting for the three linear regions, were 5.0 and 7.0, respectively.

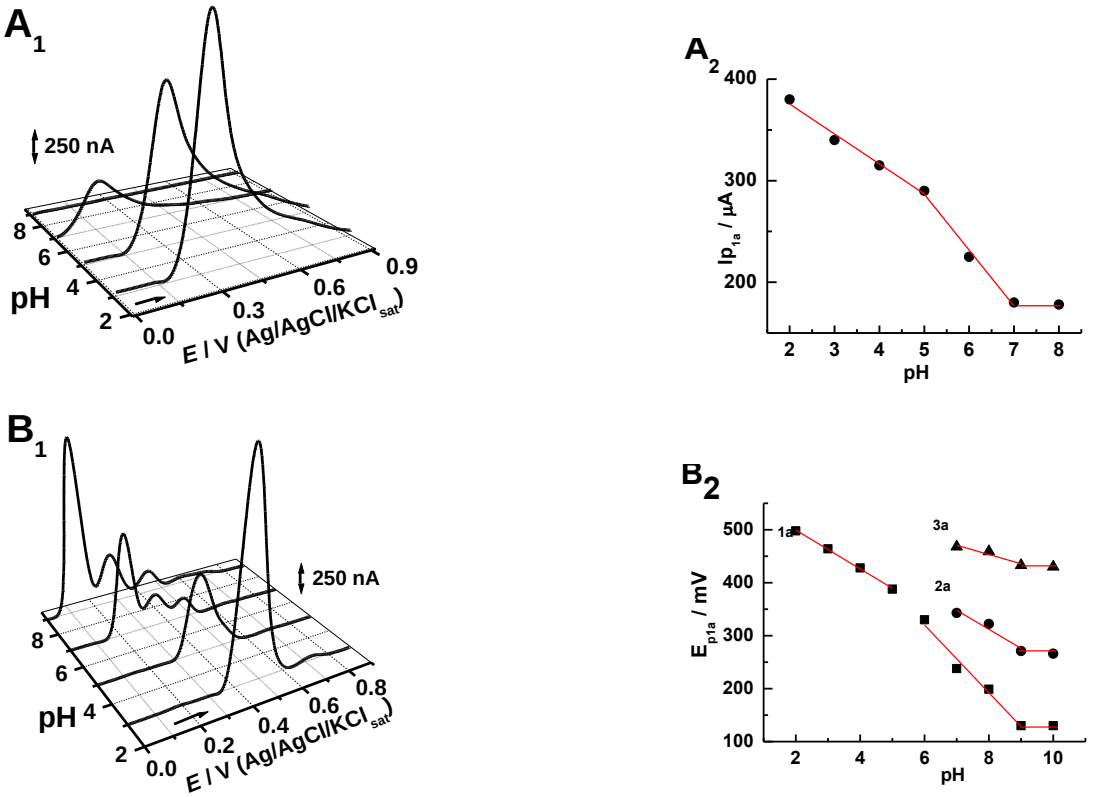

Figure 5. 3D plot of DP voltammograms with base-line corrected and plot of E_{p1a} vs pH: (A) 10 mM oenothein B and (B) 10 mM ellagic acid.

DP voltammograms obtained in the ellagic acid solution showed the same three linear regions in the E_p vs pH plot for E_{p1a}. The linear fitting for the range of 2.0 □ pH □ 5.0 and 6.0 □ pH □ 9.0 were: E_{p1a}/V = 572.6 - 36.6 pH and E_{p1a}/V = 703 – 63.9 pH. The pk_1 and pk_2 values were 4.8 and 9.0, respectively. Both values are in disagreement with those previously reported 5.42 and 6.76 [24]. Increasing pH, two additional defined peaks at E_{p2a} = 0.35 V and E_{pa3} = 0.45 V (pH 7.0) were observed.

These pK values are in agreement with the electrochemical oxidation of some phenolic compounds in which coupling reactions, as dimmerization of the phenoxyl radical, very reactive product of phenol mono electronic oxidation, are involved. As result, new peaks can be observed, principally in alkaline medium where the production of phenoxyl radical is facilitated [24].

From the discrepancy observed between the pK_1 and pK_2 values determined for the ellagic acid in the relation to the values reported previously, in the literature it is possible to admit that the voltammetric techniques are not adequate to determine the pK of the phenolic compounds in alkaline medium, principally if the electrochemical oxidation takes to the formation of another oxidizable species via chemical coupled reactions. In these cases the potentiometric methodology seems more adequate and will produce more reliable values [24].

It is important to point out that, under the electrochemical view point, the principal difference between these two compounds is the absence of coupled chemical reactions in the oenothein B

molecule due principally to the steric effects related to the complexity of this molecule, but the electrochemical oxidation of both molecules follows some similar pathways.

Furthermore, it can be also observed from Figure 5A, that oenothein B is very unstable in alkaline medium. In fact, the anodic peak 1a decreased intensely when the pH of electrolyte solution was higher than 6.0, disappearing in pH > 8.0. Consecutive DP voltammograms performed in oenothein B solution after different times showed that, also in pH 5.0, the concentration of the electro active species decreases with the time, Figure 6.

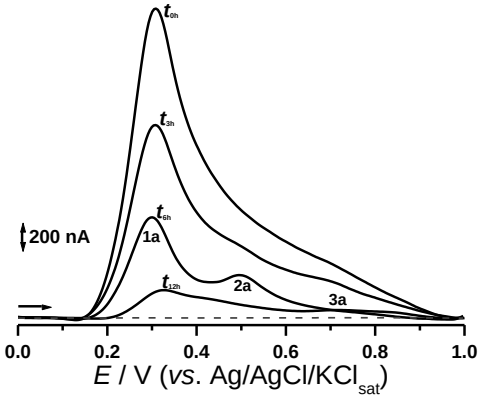

Figure 6. Sucessive DP voltammograms obtained voltammograms obtained in 0.1 M phosphate buffer solution, pH 5.0, containing for 10 mM Oenothein B after different times, *t* (0, 3, 6 and 12h). Pulse amplitude of 50 mV and scan rate of 10 mV s^{-1}.

3.3 Square Wave Voltammetry

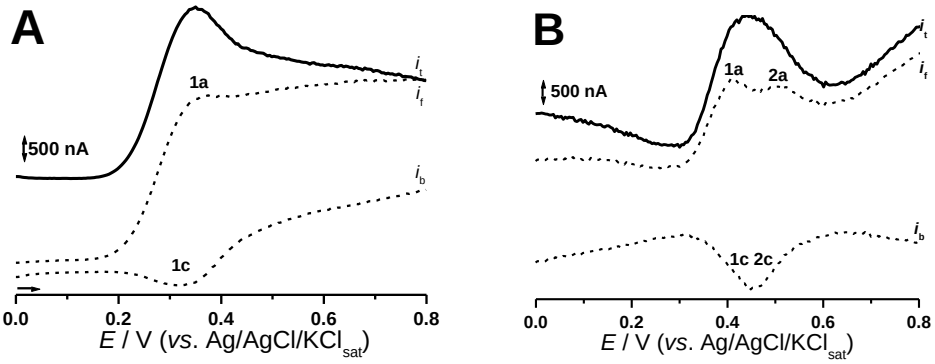

Figure 7. SW voltammograms obtained at GC electrode in 0.1 M PBS containing: 10 mM Oenothein B (**A**) and 10 mM ellagic acid (**B**). Experimental Conditions: Pulse amplitude of 60 mV, f = 30 Hz; ΔEs = 4 mV, ν_{eff} = 120 mV s^{-1}. I_t – total, I_f – forward and I_b – backward current.

Figures 7A and 7B show the SW voltammograms obtained in 0.1 M PBS, pH 5.0, containing 10 mM oenothein B or 10 mM ellagic acid. The reversibility of the first electrochemical oxidation process for oenothein B and ellagic acid at E_{p1a}/E_{p1c} redox couple 1a/1c was confirmed by the forward and backward components of the total current obtained in the square wave voltammogram. Note that in

the ellagic acid solution, Figure7B, the first peak potential, E_{p1a}, shifted to more positive direction and the peaks 1a and 2a could be observed independently.

4. CONCLUSIONS

Cyclic voltammograms showed two well defined anodic peaks, peak 1a and 2a were detected at + 0.36 V (E_{p1a}) and + 0.55 V (E_{p2a}), but high decrease in the anodic current peaks were observed in the second cycle. Reversing the scan direction, no or very discrete cathodic peaks were observed. When oenothein B was substituted by ellagic acid, the behavior was a little different: the anodic current peaks also decreased at the second cycle, but two low current intensity cathodic shoulders were detected at + 0.42 V (E_{p1a}) and + 0.54 V (E_{p2a}), which better defined in the second scan.

Differential pulse voltammograms showed that for both compounds the first oxidation peak potential shift to less positive potential increasing pH. E_{p1a} – pH plots for both compounds showed three linear regions, with appearance of new oxidation peaks at pH > 6.0 for ellagic acid. These new oxidation peaks were attributed to the production of new electroactive species, produced by coupled chemical reactions involving the eventual dimmerization of phenoxyl radicals. These coupled chemical reactions were not observed for enothein B due to steric effects, characteristic of the complexity of this molecule.

E_{p1a} – pH plots were used to determine de values of pK_1 and pK_2 of oenothein B and ellagic acid, but due the parallel chemical reactions, the data obtained for ellagic acid are not reliable, principally for pK_2 determination.

The SWV showed that the first oxidation process is reversible for both compounds, but the peak potential is less positive for oenothein B. In the pH range of 5.0 – 7.0, where E_{p1a} changes 55 mV/pH, the electrochemical oxidation should follow the general process involving 2 proton and 2 electrons in the galloyl group, where the steric effects due the molecule complexity could be less pronounced. In the ellagic acid molecule, the slope of E_{p1a} – pH plots indicated that the electrochemical oxidation involves a mixed process in all pH range and therefore, chemical coupled reactions cannot be discarded.

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