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On the adsorption and electrochemical oxidation of flavones apigenin and acacetin at a glassy carbon electrode

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ABSTRACT

The electrochemical behaviour of flavones apigenin and acacetin was studied over a wide pH range using a glassy carbon electrode. The oxidation of both compounds is irreversible, pH dependent and occurs with the transfer of one electron and one proton from the hydroxyl group in their structures. The formation of two redox products that are reversibly oxidised was observed. The adsorption of apigenin and acacetin at the glassy carbon surface was also evaluated. It has been shown that, depending on solution concentration and/or adsorption time, the molecules can adopt different orientations influencing the peak potentials and their redox behaviour. The redox properties of apigenin and acacetin were discussed in respect to their conformations, functional groups attached to their structures, and to the isomer genistein.

1. Introduction

Flavonoids are natural phytochemicals [1] that provide the essential link between diet and the prevention of chronic disorders [2]. Flavonoids exert beneficial biological effects [3] such as lowering the incidence of cardiovascular diseases [4], attenuation of neurodegenerative effects [5], and anti-cancer properties [6], among others. Their bioactivity is related to the nature and position of the substituents on their ring system [7], while the *in vivo* biotransformation may result in a gain or loss of activity [8]. The understanding of structure–activity relationships of flavonoids emerges as an important aspect for predictive determination of their biological mode of action [9].

Apigenin (5,7,4′-trihydroxyflavone) and acacetin (5,7-dihydroxy-4′-methoxyflavone) Scheme 1A and B, respectively, are two flavones mostly found in parsley, celery and chamomile. Both compounds present very low intrinsic toxicity and cancer chemopreventive and antiproliferative activities against different tumour cells [10]. Various studies have shown that acacetin and apigenin induce apoptosis in cancer cell lines through the inhibition of different enzymes systems [11,12], offering opportunities for screening new synthetic drugs for cancer treatment [13]. Also, the biological effects of apigenin and acacetin have been related to antioxidant mechanisms. However, as redox agents, they can play the role of oxidants, thus having a negative impact [14,15]. The knowledge on the oxidation mechanism of apigenin and acacetin is important for understanding their biochemical properties.

The redox behaviour of several flavonoids [16–19], their metal chelating [20,21], reactive oxygen species scavenging [22] as well as DNA interaction properties [23,24] have been studied by electrochemical methods. The research showed some general trends in their electron-donating abilities [16–18]. It was demonstrated that the hydroxyl groups in the B-ring are more easily oxidised than those in the A-ring [18], and that the oxidation potentials can be related to their antioxidant power [24]. A low oxidation potential indicates a high antioxidant capacity, usually observed for compounds containing a cathecol group which can be reversibly oxidised. The absence of the cathecol group affects the oxidation mechanism [16]. From this point of view, the detailed differences between flavonoids structure needs to be further explored in order to understand their mechanism of action.

The oxidation of apigenin was previously reported when comparing the antioxidant activity of flavones and isoflavones [25], but these studies were carried out by cyclic voltammetry in specific conditions of pH and potential range and/or for the development of methodologies for its analytical determination [19]. On the other hand, nothing is known about the electrochemical behaviour of acacetin. In fact, most studies related to apigenin and acacetin dealt with their analytical determination by chromatography [26–29].

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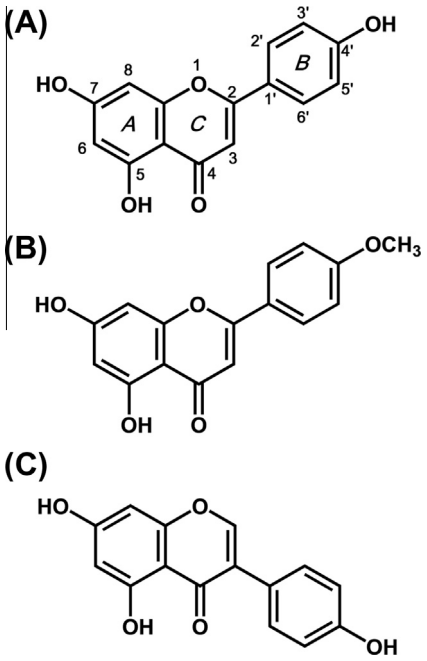


Scheme 1. Chemical structures of: (A) apigenin, (B) acacetin and (C) genistein.

In this context, the aim of this work was a qualitative, systematic and comparative study on the oxidation of apigenin and acacetin over a wide pH range using a glassy carbon electrode and electrochemical techniques, cyclic, differential pulse and square wave voltammetry. Special attention was paid to the adsorption of apigenin and acacetin at the electrode surface since this process has an important effect on their electrochemical behaviour [17,18,24]. Also, the electrochemical properties of apigenin isomer genistein were reviewed in order to understand the influence of structural features on their electrochemical behaviour. The comparative electrochemical study of flavonoids in general, and apigenin, acacetin and genistein in particular, allow the determination of their redox mechanism and contribute to a qualitative understanding of their structure–activity relationships.

2. Experimental

2.1. Materials and reagents

Apigenin, acacetin and genistein were obtained from Extrasynthèse (Genay, France) and used without further purification. Stock solutions of 5 mM were prepared in ethanol-deionised water (50/50, v/v) 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 $\leqslant 0.1\ \mu S\ cm^{-1}$) Table 1.

2.2. Voltammetric parameters and electrochemical cells

Voltammetric experiments were carried out using an Ivium potentiostat running with Ivium software version 2.038, Ivium Technologies, The Netherlands. Measurements were carried out using a three-electrode system in a 0.5 mL one-compartment electrochemical cell (Cypress System Inc., USA). Glassy carbon electrode (GCE, $d = 1.0$ mm) was the working electrode, Pt wire

Table 1
Supporting electrolytes, 0.1 M ionic strength.

pH	Composition
2.0	HCl + KCl
3.4	HAcO + NaAcO
4.3	HAcO + NaAcO
5.6	HAcO + NaAcO
7.0	$NaH_2PO_4 + Na_2HPO_4$
8.0	$NaH_2PO_4 + Na_2HPO_4$
9.2	$NH_3 + NH_4Cl$
10.0	KCl + NaOH
12.0	KCl + NaOH

the counter electrode and the Ag/AgCl ($3\ mol\ L^{-1}$ KCl) reference electrode.

The pH measurements were carried out with a Crison micropH 2001 pH-metre with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1 °C) and microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA).

The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 100 ms, potential step 2 mV and a scan rate of $5\ mV\ s^{-1}$. For square wave (SW) voltammetry were: pulse of 50 mV, frequency of 25 Hz and a potential increment of 2 mV, corresponding to an effective scan rate of $50\ mV\ s^{-1}$ were used.

The GCE was polished using diamond particles of 3 μm (Kemet, UK) 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.

2.3. Acquisition and presentation of data

All the voltammograms presented were background-subtracted and baseline-corrected using the automatic function included in the Ivium software [16].

All measurements have been performed in triplicate and the results presented are the average of three independent experiments.

Conformational optimisation of the protonated compounds was done using the MOPAC module and the semi-empirical method for the quantum calculation of molecular electronic structure Austin Model 1 (AM1), with an open shell (unrestricted) wave function, included in the Chem 3D Ultra 8.0 software from ChemOffice 2004.

Origin Pro 8.0 from OriginLab Corporation was used for the presentation of all the experimental data reported in this work.

3. Results

3.1. Cyclic voltammetry

Apigenin is a flavone that contains three hydroxyl groups: one at position 4′ in the B ring forming a phenol structure, and two in the A ring at positions 5 and 7 corresponding to a resorcinol structure Scheme 1A.

Cyclic voltammograms were recorded in a solution of 50 μM apigenin in pH = 4.3 Fig. 1A, between +0.00 V and maximum potential limit of +1.20 V. On the first positive-going scan of the first voltammogram two overlapping peaks $1'_a$ and 1_a occurred at $E_p1'_a = +0.78$ V and $E_p1_a = +0.85$ V, respectively, and at higher positive potential peak 2_a appeared at $E_p2_a = +1.07$ V.

Reversing the scan direction, on the negative-going scan of the first voltammogram, two small cathodic peaks: 3_c, at $E_p3_c = +0.42$ V, and 4_c, at $E_p4_c = +0.22$ V, appeared. These peaks

are due to the reduction of apigenin oxidation products formed at the electrode surface during the first voltammetric scan. On the second voltammogram in the same conditions without cleaning the electrode surface the corresponding anodic peaks 4_a, at E_p4_a = + 0.25 V, and peak 3_a, E_p3_a = +0.45 V were observed Fig. 1A. The decrease of peaks $1'_a$, 1_a and 2_a on the second voltammogram was due to the adsorption of apigenin and/or its oxidation products at the GCE surface.

A new experiment was carried out in the same conditions but the scan direction was reversed at +0.95 V, after the occurrence of peaks $1'_a$ but before peaks 1_a and 2_a Fig. 1A. In these conditions peaks 4_a and 4_c appeared showing that they are related to the product formed after apigenin oxidation at peaks $1'_a$.

Also, cyclic voltammograms were obtained for different scan rates in a 50 μM apigenin solution in pH = 4.3 Fig. 1B. Between measurements, the electrode surface was always polished in order to ensure a clean surface. Increasing the scan rate, peaks $1'_a$ and 1_a, merged and only one main oxidation peak 1_a occurred. The potential of peaks 1_a and 2_a turned more positive and their currents increased linearity with the scan rate indicating the adsorption of apigenin and its oxidation products at the electrode surface (not shown).

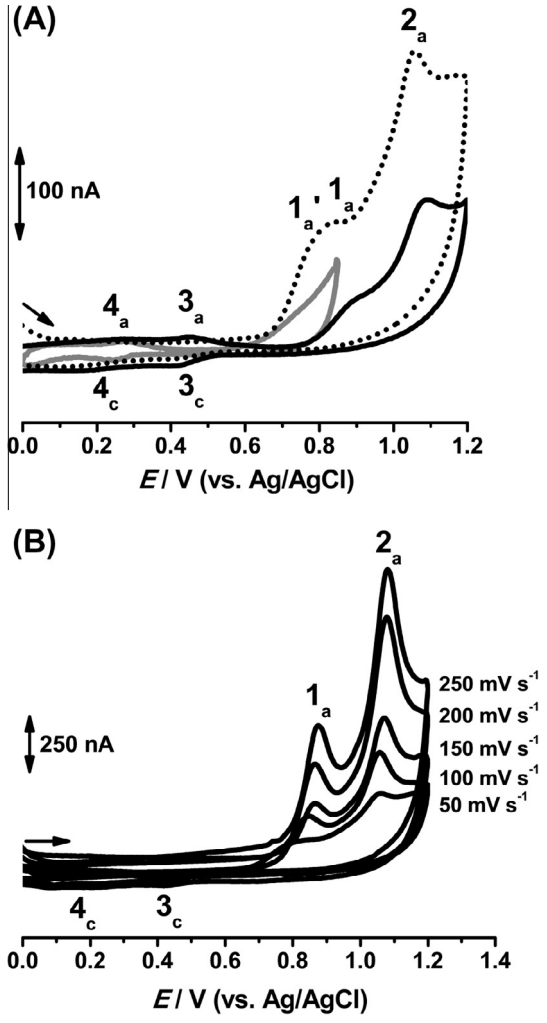


Fig. 1. Cyclic voltammograms base-line subtracted in 50 μM apigenin in pH = 4.3: (A) (dotted curve) first and third scans between +0.00 V and (black curve) +1.20 V or (grey curve) +0.95 V at v = 100 mV s^{-1} and (B) first scan at different scan rates.

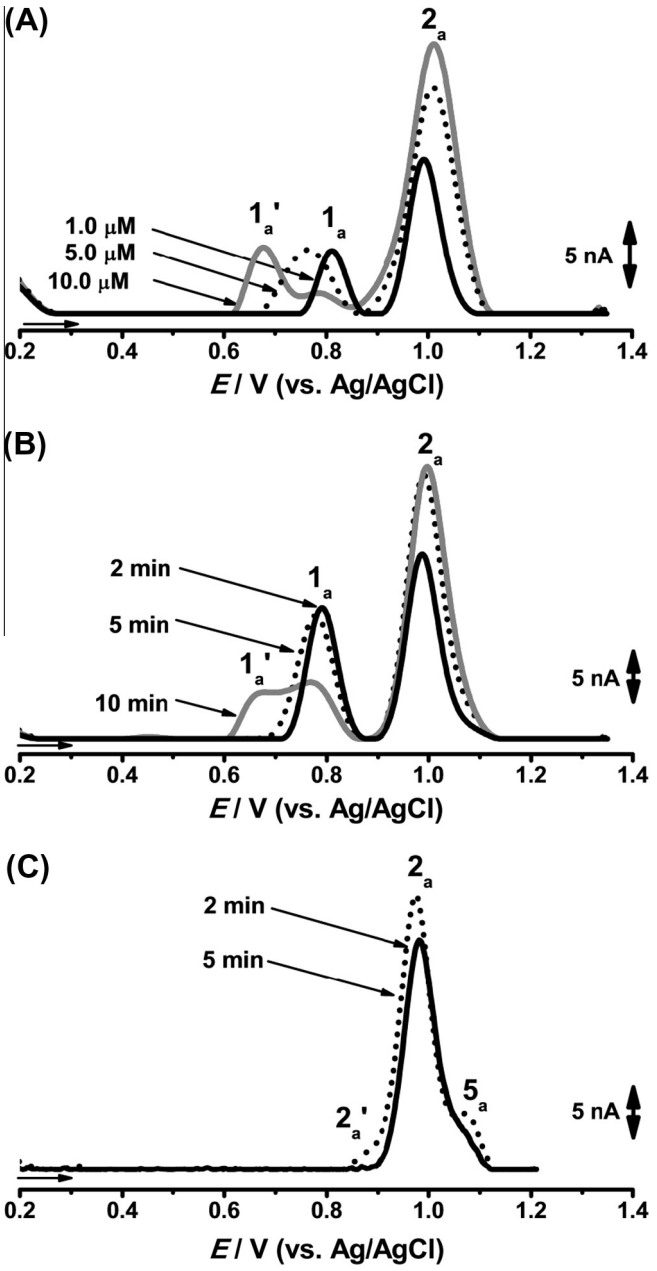


Fig. 2. DP voltammograms base-line corrected: (A) first scans in solutions of 1.0, 5.0 and 10.0 μM apigenin in pH = 4.3, and in pH = 4.3 after adsorption during different times from solutions of 5 μM: (B) apigenin and (C) acacetin.

3.2. Differential pulse voltammetry

3.2.1. Apigenin

DP voltammograms were recorded in solutions of different concentrations of apigenin in pH = 4.3 Fig. 2A. For 1 μM apigenin, two consecutive charge transfer reactions were observed: peak 1_a at E_p1_a = + 0.82 V and peak 2_a at E_p2_a = + 0.99 V Fig. 2A. With increasing the solution concentration, peak 1_a became broader, and the DP voltammogram recorded in 10 μM apigenin showed a new anodic peak $1'_a$ at lower potential values $E_p1'_a$ = +0.68 V.

The adsorption of apigenin at the electrode surface was also studied Fig. 2B. The GCE was held during different periods of time in a solution of 5 μM apigenin in pH = 4.3. Then, the electrode was washed with a jet of deionised water and placed in the electrochemical cell containing only the supporting electrolyte pH = 4.3

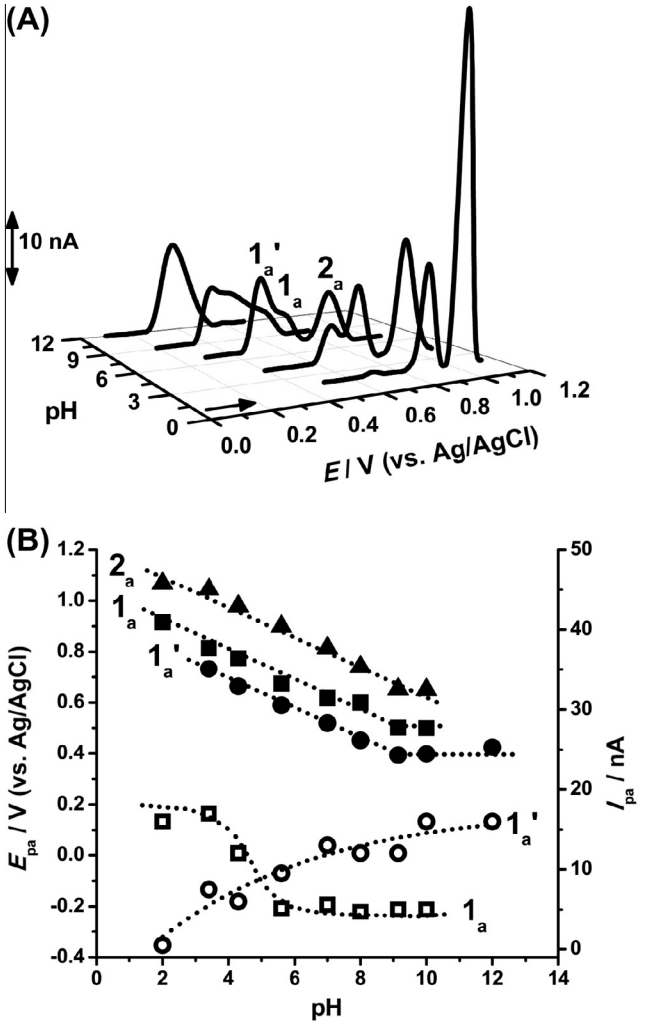


Fig. 3. (A) 3D plot of DP voltammograms baseline-corrected in 10 μM apigenin function of the pH of the supporting electrolyte and (B) plot of E_{pa} of peaks (●) $1'_a$, (■) 1_a and (▲) 2_a and of I_{pa} of peaks (○) $1'_a$ and (□) 1_a vs. pH of the supporting electrolyte.

where DP voltammetry was performed. The voltammogram obtained for 2 and 5 min of adsorption, showed peaks 1_a and 2_a. After 10 min of adsorption, peak $1'_a$ appeared at a lower potential Fig. 2B. A similar behaviour was observed for electrolytes with different pH values (not shown).

The pH effect was investigated in solutions of 10 μM apigenin over a wide pH range from 2.0 to 12.0 Fig. 3A.

For strong acid electrolytes, peaks 1_a and 2_a occurred on the first scan Fig. 3A. For pH > 3.4, peak $1'_a$ appeared at lower potential values.

For electrolytes with pH < 10.0, the potential of peaks $1'_a$, 1_a and 2_a shifted to less positive values with increasing the pH of the supporting electrolyte Fig. 3A. The relationships were linear and the slope of the lines −60 mV per pH unit Fig. 3B, in agreement with the transfer of the same number of electrons and protons. The width at half-height of the peaks was around 90 mV, close to the theoretical value for the transfer of 1 electron.

For pH > 10.0, peaks $1'_a$ and 1_a potential did not depend on the pH of the supporting electrolyte, in agreement with electrochemical reactions that involve the transfer of electrons after chemical deprotonation.

Also, consecutive DP voltammograms were recorded in solutions of apigenin in electrolytes with different pH values Fig. 4A and B. On the first DP voltammogram in pH = 4.3, peaks $1'_a$, 1_a and 2_a were observed Fig. 4A. In successive DP voltammograms, peaks 3_a and 4_a due to apigenin oxidation products appeared at lower potentials. At the same time, peak $1'_a$ disappeared and peaks 1_a and 2_a occurred with lower currents while their potential shifted. A similar electrochemical behaviour was observed in other electrolytes Fig. 4B, although the current of peaks 3_a and 4_a decreased with increasing pH.

3.2.2. Acacetin

Acacetin contains two hydroxyl groups in the A ring at positions 5 and 7 forming to a resorcinol type structure Scheme 1B.

The adsorption of acacetin at the electrode surface was studied Fig. 2C. The GCE was held during different periods of time in a solution of 5 μM acacetin in pH = 4.3. Then, the electrode was washed with water and placed in the electrochemical cell containing only the supporting electrolyte pH = 4.3. The DP voltammogram recorded in after 2 min of adsorption, showed peaks 2_a and 5_a. For longer adsorption times, peak $2'_a$ occurred at a lower potential value Fig. 2C. A similar behaviour was observed in other supporting electrolytes.

DP voltammograms were recorded in solutions of 10 μM acacetin in electrolytes with different pH values Fig. 5A.

For acid electrolytes two consecutive charge transfer reactions at peaks 2_a and 5_a were observed, whereas for pH > 4.3 peak $2'_a$ also occurred at lower potential values Fig. 5A and B.

For electrolytes with pH < 10.0, peaks $2'_a$, 2_a and 5_a were pH dependent and their potentials turned less positive with increasing pH Fig. 5B. For peaks $2'_a$ and 2_a the linear relationships had a slope of −60 mV per pH unit. The width at half-height of the peaks was around 90 mV, in agreement with the transfer of 1 electron and 1 proton. For peak 5_a the slope of the line was about −30 mV per pH unit showing that the mechanism of this oxidation process involves a number of electrons that is double the number of protons.

For pH > 10.0, peak $2'_a$ did not depend on the pH of the supporting electrolyte.

Consecutive DP voltammograms were also recorded in solutions of acacetin in electrolytes with different pH values Fig. 4C and D. On the first DP voltammogram in pH = 4.3, peaks 2_a, $2'_a$ and 5_a were observed Fig. 4C. In successive DP voltammograms, peaks 3_a and 4_a due to the oxidation of acacetin redox products appeared at lower potential values Fig. 4C. In pH = 7.0 Fig. 4D, the anodic peak $2'_a$ occurred with higher currents at lower positive potential values. By recording successive voltammograms, the peaks correspondent to acacetin oxidation products appeared.

3.2.3. Genistein

Genistein is an isomer of apigenin Scheme 1C. The redox mechanism of genistein has been previously studied in detail [16], but its electrochemical behaviour was reviewed in order to gain insights into the redox mechanism of apigenin.

On the first DP voltammogram recorded in a solution of 5 μM genistein in pH = 4.3 Fig. 4E, three consecutive charge transfer reactions were observed: peak 1_a at E_p1_a = +0.59 V, peak $2'_a$ at $E_p2'_a$ = +0.87, and peak 2_a at E_p2_a = +0.98 V. On the second voltammogram recorded in the same conditions without cleaning the GCE surface, peaks 4_a and 5_a due to the oxidation of genistein redox products appeared at E_p3_a = +0.40, E_{p4a} = +0.20 V, respectively. At the same time, the decreased of peak 1_a, $2'_a$ and 2_a was due to the adsorption of the genistein redox products reducing the available electroactive area. A similar behaviour was observed in other electrolytes Fig. 4F.

Genistein oxidation is pH-dependent, occurs in three consecutive steps, each involving the transfer of one electron and one proton from the correspondent hydroxyl groups in its structure [16].

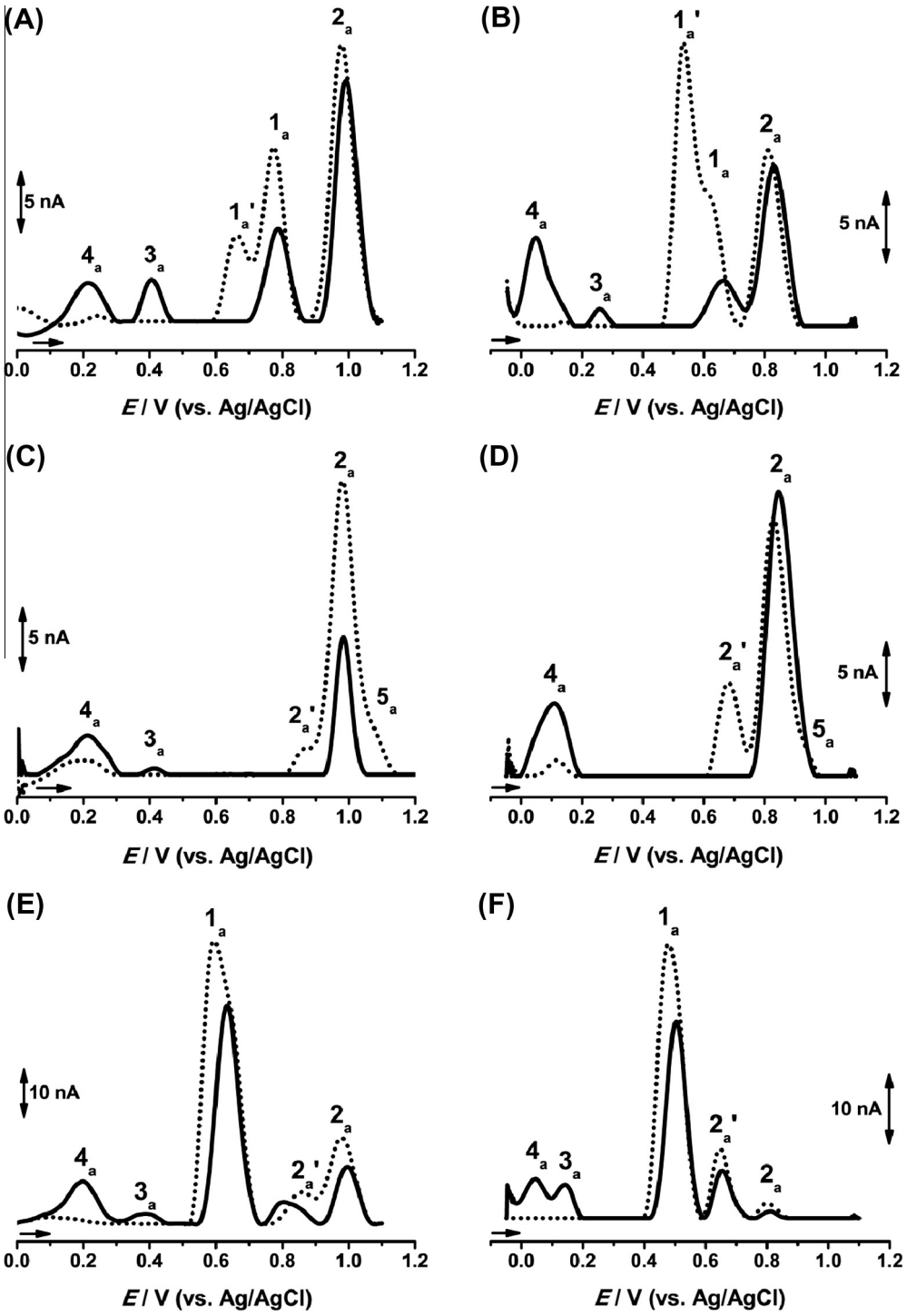


Fig. 4. DP voltammograms baseline-corrected in 10 μM: (A and B) apigenin, (C and D) acacetin and (E and F) 5 μM genistein in pH: (A, C, E) 4.3 and (B, D, F) 7.0; (dotted curve) first and (black curve) second scans.

3.3. Square wave voltammetry

Successive SW voltammograms were recorded in a solution of 10 μM apigenin in pH = 4.3 Fig. 6. On the first scan the anodic peak $1'_a$ at $E_p1'_a$ = +0.73 V, peak 1_a at E_p1_a = +0.79 V and peak 2_a at E_p2_a = +1.02 V, occurred. By plotting the forward and backward components of the total current, a small reduction current correspondent to peak $1'_a$ was observed. However, the irreversibility of apigenin oxidation at peaks 1_a and 2_a was observed since only anodic currents occurred on the forward component and no cathodic correspondent on the backward one Fig. 6A.

By increasing the number of scans in solution, without cleaning the GCE surface, peaks 3_a and 4_a appeared at E_p3_a = +0.22 V and at E_p4_a = +0.43 V Fig. 6B. The deconvolution of the total current recorded in these conditions has shown the reversibility of peaks 3_a and 4_a since by plotting the forward and backward components the reduction and oxidation currents were equal and occurred at the same potential value Fig. 6B.

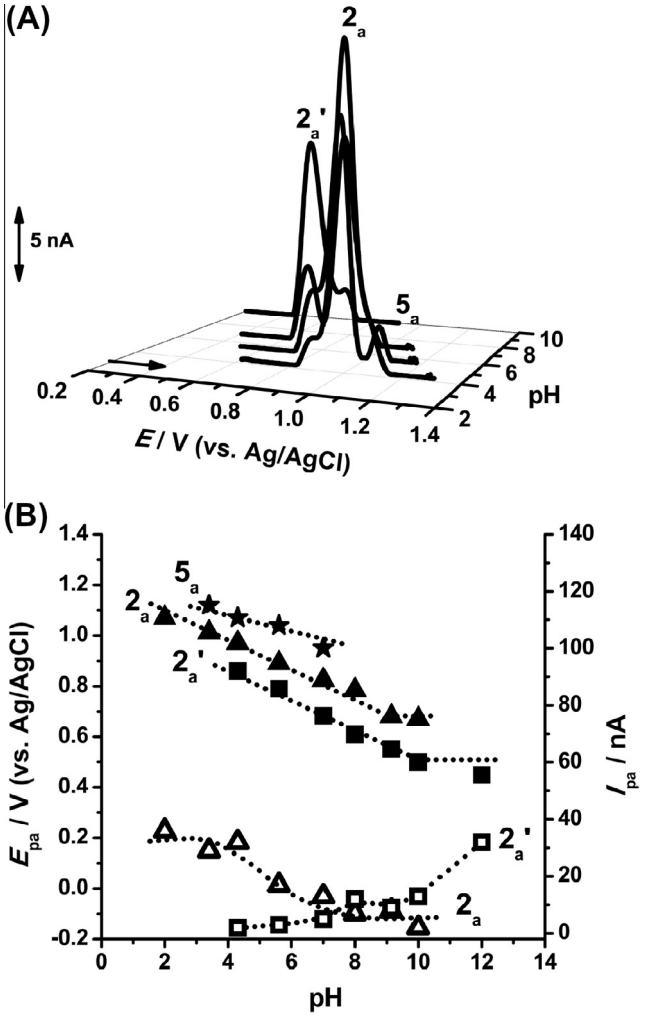


Fig. 5. (A) 3 D plot of DP voltammograms baseline-corrected in 10 μM acacetin function of the pH of the supporting electrolyte and (B) plot of E_{pa} of peaks (■) $2'_a$, (▲) 2_a and (★) 5_a and of I_{pa} of peaks (□) $2'_a$ and (△) 2_a vs. pH of the supporting electrolyte.

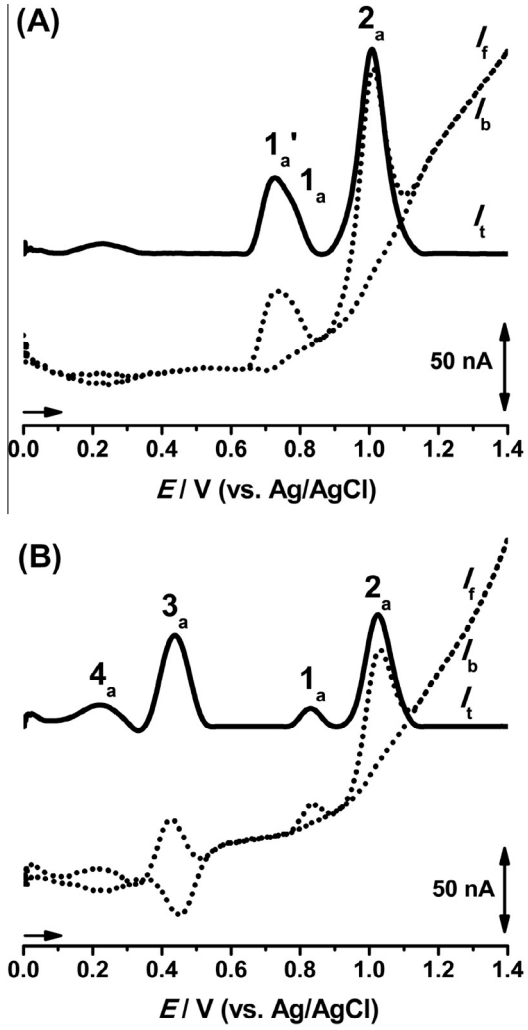


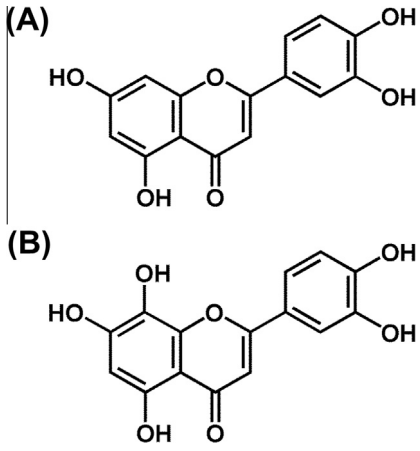
Fig. 6. SW voltammograms baseline-corrected in 10 μM apigenin in pH = 4.3: (A) first and (B) second scans; I_t, I_f and I_b – total, forward and backward currents, f = 25 Hz, ΔE_s = 2 mV for v = 50 mV s^{-1}.

4. Discussion

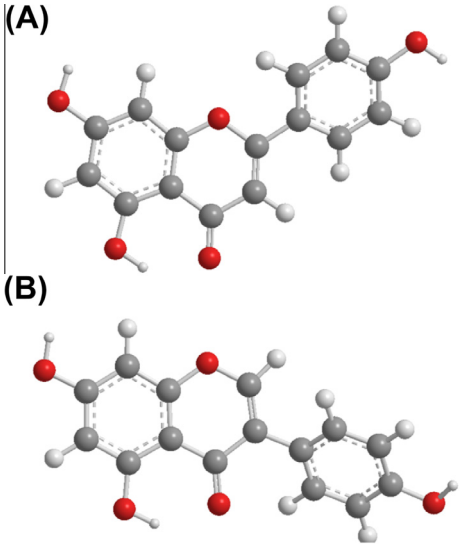
Apigenin and genistein present a hydroxyl group in the ring B Scheme 1A and C, which is oxidised at peak 1_a, whereas acacetin lacks this hydroxyl group Scheme 1B, and peak 1_a is absent Fig. 4. The cyclic voltammograms recorded between +0.00 V and maximum potential values correspondent to peak 1_a Fig. 1A, have shown the occurrence of one oxidation product that undergoes reversible redox reactions, peaks 4_c–4_a. The oxidation of the hydroxyl group in ring B at peak 1_a involves the formation of an *ortho*-quinone specie Scheme 2A, since the *para* position is occupied [16,30].

The voltammograms recorded in solutions with different concentrations of apigenin or after its adsorption during different times have shown the occurrence of a new peak $1'_a$ at lower potential values Fig. 2. Contrary, the oxidation of genistein has shown only peak 1_a. The occurrence of peak $1'_a$ is explained considering different orientations of apigenin molecules at the GCE surface [31,32].

Apigenin presents a more planar conformation Scheme 3A, when compared to genistein which shows a larger dihedral angle [25] between the planar A–C rings moiety and ring B Scheme 3B. It is proposed that, for low bulk concentrations, apigenin molecules adsorbed with the rings system parallel to the electrode surface and only peak 1_a was observed Fig. 2A and B. By increasing the solution concentration Fig. 2A and B, stacking and lateral interactions became more effective and led to reorientation of the



Scheme 2. Proposed redox products of apigenin after oxidation at peak: (A) 1_a and (B) 2_a.



Scheme 3. Conformation of: (A) apigenin and (B) genistein.

molecules in a conformation that facilitates their oxidation at peak $1'_a$ Fig 2. Contrary, the reorientation of genistein molecules is impeded by steric effects imposed by the tilted A–C moiety [25], and the oxidation of genistein gave rise only to peak 1_a. Nevertheless, after scanning the potential, the apigenin molecules are rearranged on the electrode surface and on the second scans, peak $1'_a$ did not appear anymore Fig. 4A and B.

On the other hand, it has been observed that peak $1'_a$ current increased with the pH Figs. 3A and 4B, whereas peaks 1_a and 2_a progressively decreased and disappeared for electrolytes with pH > 10.0 Fig. 3A and B. This effect is due to the pH-dependent adsorption of apigenin and/or its oxidation products at the electrode surface. In fact, with increasing pH, apigenin became ionised (deprotonised) and this process altered its hydrophobicity and consequently the adsorption at the GCE surface.

All compounds present two hydroxyl groups forming a resorcinol structure in ring A, which is oxidised at higher potential values than ring B [16,30]. The voltammograms recorded in solutions of acacetin, have shown the occurrence of two consecutive peaks $2'_a$ and 2_a Fig. 5, which are associated with the oxidation of each hydroxyl group in its structure. In agreement, the oxidations of these hydroxyl groups involved the formation of *ortho*- and/or *para*-quinone [30] Scheme 2B, as two peaks correspondent to acacetin oxidation products were observed Fig. 4C and D.

The DP voltammograms recorded in solutions of acacetin in acid media have shown peak 5_a at higher potential values Fig 5. It is proposed that this peak is related to the oxidation of a specie formed by the chemical reaction between the initial radical cation with an acacetin molecule through the methoxyl group on the B-ring [16]. The absence of peak 5_a in solutions of apigenin Figs. 3 and 4A and B, could be explained by the steric effects that impede a similar reaction.

Apigenin and its isomer genistein are discriminated by the position of ring B relative to ring C. Although their oxidation mechanisms are similar, the structural difference influences their electrochemical behaviour. It is proposed that the lower oxidation potential of genistein relative to apigenin Fig 4, is due to the influence [33] of the oxygen atom at position 4 in ring C on the electroactive centre Scheme 1. In the case of genistein, the electronegativity of the oxygen atom in ring C partially displaces the delocalised electron cloud in ring B [33], facilitating the oxidation. Contrary, a higher distance between the oxygen atom in ring C and the hydroxyl group in ring B of apigenin could be responsible for a higher oxidation potential value.

5. Conclusions

The redox behaviour of the flavones apigenin and acacetin, was investigated using a glassy carbon electrode and cyclic, differential pulse and square wave voltammetry.

The oxidation of both compounds is an irreversible, pH dependent process that occurs in a cascade mechanism, each step involving the transfer of one electron and one proton from the hydroxyl groups attached to their structures.

In acid and neutral electrolytes, the oxidation process is strongly influenced by the adsorption at the glassy carbon electrode surface. It has been shown that different orientations of adsorbed molecules influence the peaks potential and the redox behaviour of both compounds. In strong alkaline media, only one pH independent charge transfer reactions was observed. The oxidation of apigenin and acacetin involves the formation of two oxidation products that undergo reversible redox reactions.

The electrochemical behaviour of genistein was reviewed and allowed understanding the effect of the position of the aromatic rings on the electrochemical behaviour of apigenin and acacetin. The redox properties of apigenin and acacetin were discussed in respect to their conformations and functional groups attached to their structures.

Acknowledgments

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