Int. J. Electrochem. Sci., 12 **(2017)** 7616 – 7637, doi: 10.20964/2017.08.79

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Influence of Chemical Structure of Some Flavonols on Their Electrochemical Behaviour

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Received: 21 April 2017 / *Accepted:* 15 June 2017 / *Published:* 12 July 2017

The electrochemical behaviour of three structurally related flavonols, quercetin, morin, and rutin was studied by cyclic, differential pulse, and square-wave voltammetry methods. The study reveals that their electrochemical behaviour strongly depends on their chemical structure and electronic properties, particularly on the presence of electron-donating -OH groups, i.e. their numbers and position on rings A, B, and C in the structure of these flavonols. The important factors of the electrochemical oxidation behaviour of flavonols are as follows. (i) The presence of two electron-donating -OH groups on the B ring in the *ortho*-position. (ii). The 2,3-double bond in conjugation with a 4-oxo group on the C ring. (iii) The electron donating 3-OH group on the C ring, and (iv) the electron-donating 5-OH and 7-OH groups on A ring. Quercetin satisfies all of the requirements mentioned above and therefore has the best electron-donating properties of all investigated flavonols. The first oxidation peak of quercetin (peak A1) corresponds to the reversible oxidation of 3',4'-OH groups (catechol moiety) at the B ring to the *ortho*-quinone structure by two-electron-two-proton ($2e^-$-$2H^+$) process. This electrochemically active and unstable *ortho*-quinone species then undergoes chemical rearrangements or addition reactions, indicates an electrochemical-chemical (EC) reaction mechanism. At higher anodic potential the -OH group at position 3 of ring C was oxidized (peak A2), by the reversible one-electron-one-proton reaction. In this study, it was for the first time observed that the second reduction peak of quercetin (peak C2) corresponds to the 3-OH group on ring C. The hydroxyl groups at position 5 and 7 at ring A have significantly smaller electron-donating effect than -OH groups at ring B, and therefore were oxidized at higher anodic potentials (peak A3). This oxidation is an irreversible process. Morin with *meta*-2',4'-dihydroxyl groups (resorcinol moiety) shows the higher value of oxidation potential of peak A1 than quercetin, indicating that oxidation of 2',4'-OH groups on ring B of morin to quinone structure is more difficult in comparison to that of quercetin. This fact clearly shows the importance of the presence of two hydroxyl groups in the *ortho*-diphenolic arrangement on the ring B of flavonols. The first oxidation process of morin is an one-electron-one-proton reversible reaction, which proceeds in an EC mechanism. The oxidation peak of morin A3 should be associated with the oxidation of 5,7-dihydroxyl moiety of ring A, which oxidation occurs at very high oxidation potentials. This oxidation is an irreversible process because no reduction peak was observed. The absence of oxidation peak A2 could be explained possibly by the formation of hydrogen bond between the 3-OH group and the oxygen at position C-4 on the ring C. Rutin (quercetin-3-*O*- rutinose) shows the highest oxidation

potential of peak A1 of all investigated flavonols. Such behaviour reflects the influence of glycosylation with rutinose on position 3 of the C ring. Therefore, an oxidation process was blocked, what significantly decreases the strength of delocalization of electrons from B ring, in comparison to that of quercetin. In concordance with this fact, the oxidation of B ring of rutin was observed at significantly higher potentials. This result shows how important is the role of 3-OH group in the C ring on the electrochemical properties of flavonols. The first redox couple of rutin (peaks A1 and C1) corresponds (as in the case of quercetin) to the oxidation of 3',4'-dihydroxy groups on the B ring of rutin and the reduction of the 3',4'-quinone, respectively. This reaction is a reversible process proceeds through EC reaction mechanism. The oxidation peak of rutin A3 should be associated with oxidation of 5,7-dihydroxyl moiety of ring A. This oxidation is an irreversible process because no reduction peak was observed. The absence of oxidation peak A2 could be explained by the presence of rutinoside group without electrochemical activity, at position 3 of C ring of rutin. The results of present study give some new information about the electrochemical oxidation/reduction processes of investigated flavonols. (i) For the first time was observed the presence of cathodic reduction peak of quercetin (peak C2) which corresponds to 3-OH group at C ring. (ii) The reversibility of the first oxidation process of morin (peaks A1 and C1) was clearly demonstrated. (iii) It was clearly shown how important is the role of -OH groups (i.e. their number and their position on rings A, B, and C) on the electrochemical behaviour of investigated flavonols.

Keywords: Electrochemical behaviour, Quercetin, Morin, Rutin, Chemical structure

1. INTRODUCTION

Flavonols are one of the subgroups of the large family of natural polyphenolic compounds called flavonoids. The other main subgroups of flavonoids are flavanols (e.g., catechin), flavones (e.g., apigenin), isoflavones (e.g., genistein), flavanones (e.g., naringenin), and anthocyanidins (e.g., cyanidin). Chemically, flavonols (as also other flavonoids) have the characteristic 15-carbon skeleton (C6-C3-C6). This structure has been constituted by two benzene rings (catechol B ring and resorcinol A ring) joined together by a 4-pyrone heterocyclic ring C. Flavonols differ from many other flavonoids by the presence of 2,3-double bond and the 4-oxo group in the C ring. From flavones, they differ in the presence of one additional -OH group at position 3 in the C ring. Therefore, flavonols have a characteristic 3-hydroxyflavone backbone. Additionally, the 3-OH group can be glycosylated by different sugars, what significantly increases the number of flavonol isomers.

Flavonols were the most common flavonoids in many plants, such as fruits (e.g. apples, various berries, pomegranate), vegetables (broccoli, red and white onion, tomato, spinach), cocoa and chocolate, but also contained in many beverages (such as green and black teas and red wines). Quercetin is the most widely consumed flavonols (and also flavonoid) in the human diet [1-2]. Due to the widespread use of fruits, vegetables, and beverages in the human diet, the positive effects of flavonols on human health were extensively studied. Numerous epidemiological studies [3-5] have reported that flavonols (especially quercetin) show many different biological activities. Thus, flavonols can act as an antioxidant, anti-inflammatory agents, anticancer factors, as a regulator of different

cellular signal pathways (e.g. they have an influence on the oxidative stress damage of cells), but flavonols also have many others very beneficial effects on human health.

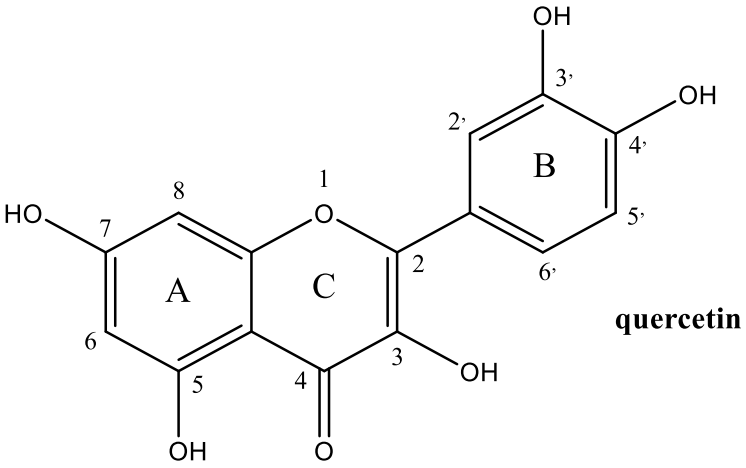
Numerous studies have shown that biological activities of flavonoids (including flavonols) are close connected with their electrochemical properties. Thus, e.g. flavonoids with lower oxidation potentials showed higher antioxidant activity [6-8]. Therefore, to better understand the complex biological activities of flavonols contained in different foods, when they were absorbed in the human body, their electrochemical behaviour must be investigated.

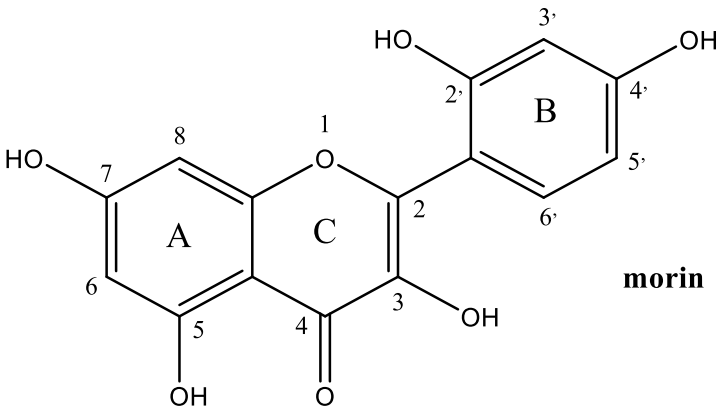
The electrochemistry of important flavonols contained in foods (quercetin, rutin, and morin) was relatively extensive investigated. Thus, many authors [9-23] were studied the electrochemical behaviour of quercetin at different experimental conditions. It could be summarized from these investigations as follows. The catechol moiety of quercetin (ring B) was oxidized first through the reversible (or quasi-reversible) two-electron-two-proton process to the *ortho*-quinone structure. The nature and mechanisms of further oxidation processes of three remaining -OH groups in rings C and A are still not-fully-understood. Authors reported a different number of additional anodic oxidation peaks observed at higher oxidation potentials (from one to three oxidation peaks) and reported different oxidation mechanisms (electrochemical or electrochemical-chemical, i.e. EC mechanism). The number of total cathodic peaks is also different in the papers mentioned above. Most authors reported only one cathodic reduction peak, while Brett et al. [11] and Timbola et al. [13] observed two cathodic peaks.

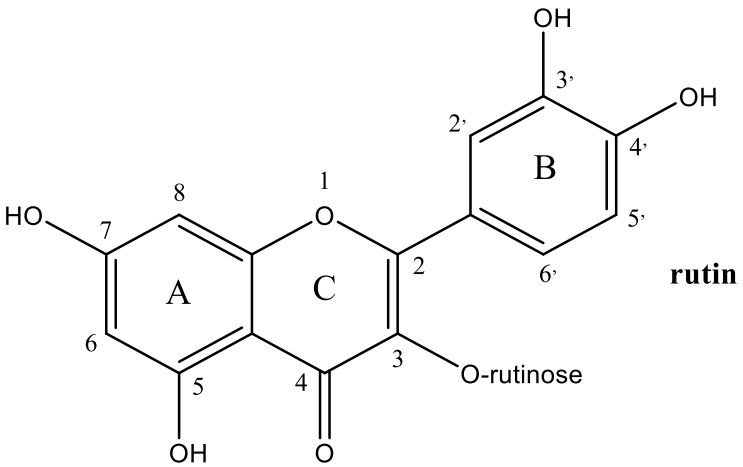
Electrochemistry of morin was also investigated in numerous papers [24-30], but still, in the published results exist many contradictions. Authors reported a different number of anodic oxidation peaks (from one to two peaks), with very different assignation of these peaks. Also, the number of observed cathodic peaks was different, from results where was not observed cathodic peak (i.e. the process was irreversible) up to results with two observed cathodic peaks. Especially, a big difference in oxidation mechanisms of morin was reported (from electrochemical to electrochemical-chemical, EC mechanism).

Electrochemical behaviour of rutin was also relatively extensive investigated [31-38]. Published results still live some doubts in the number of anodic and cathodic peaks and especially regarding the oxidation mechanism of rutin (electrochemical or EC mechanism).

From all above-mentioned published papers, in which sometimes very different results were reported, it is obviously that the nature and mechanisms of oxidation processes of electrochemical oxidation of quercetin, morin, and rutin are still not-fully-understood. The biggest problem in above reports is that these flavonols were investigated separately (i.e. all these flavonols were not studied in one paper) and different experimental conditions were used. Therefore, in the presented paper systematic investigation of the electrochemical behaviour of these three structurally very similar flavonols, under the same experimental conditions, was performed by different electrochemical techniques (cyclic voltammetry, differential pulse voltammetry, and square-wave voltammetry). The main point of the presented study was to explain how the small differences in the chemical structure of these three flavonols (quercetin, morin, and rutin, see Scheme 1.) influences on their electrochemical behaviour.







Scheme 1. Chemical structures of quercetin, morin, and rutin

2. EXPERIMENTAL

2.1. Chemicals

Three flavonols: quercetin dihydrate (purity of ≥98 %), rutin trihydrate (purity of ≥95 %), and morin hydrate (purity of ≥95 %) were obtained by Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Sodium acetate trihydrate and acetic acid for the preparation of buffer supporting electrolyte were purchased from Kemika (Zagreb, Croatia). Alumina powder of 0.05 μm used for polishing of glassy carbon electrode (GCE) was obtained by Buehler (USA).

Stock solutions of investigated flavonols (c=1·10^{-2} mol L^{-1}) were prepared in methanol (HPLC grade) and stored in a refrigerator at 4^{o}C. Working solutions of flavonols for electrochemical measurements were prepared by dilution of an appropriate quantity of stock solution with supporting electrolyte (0.1 mol L^{-1} acetate buffer solution of pH 3.6). Supporting electrolyte was prepared using the analytical grade chemicals and ultra-pure water obtained from Millipore Milli-Q purification system (conductivity of water ≤ 0.1 μS cm^{-1}).

2.2. Apparatus, experimental conditions and procedure

The electrochemical experiments (cyclic voltammetry, differential pulse voltammetry, and square-wave voltammetry) were performed using an EG&G Princeton Applied Research Model 273A potentiostat/galvanostat remotely controlled by the computer with electrochemical software Model 270/250 (EG&G Princeton Applied Research, USA). Measurements were performed in a standard three-electrode electrochemical cell (Metrohm, Switzerland). Glassy carbon electrode (GCE) of 3 mm diameter (model MF-2012, Bioanalytical Systems, USA) was used as the working electrode. The Pt wire was the counter electrode, and Ag/AgCl (3 mol L^{-1} KCL) electrode was reference electrode (both electrodes made by Metrohm, Switzerland).

The cyclic voltammetry (CV) was performed at 25, 50, 100 and 150 mV s^{-1} scan rates. The experimental parameters for differential pulse voltammetry (DPV) were as follows: pulse amplitude 50 mV, pulse width 70 ms, potential increment 2 mV, interval time 0.4 s, and a scan rate of 5 mV s^{-1}. Square-wave voltammetry (SWV) conditions used were: pulse amplitude 50 mV, frequency 50 Hz, potential increment 2 mV, and an effective scan rate of 100 mV s^{-1}.

Before each electrochemical measurements of flavonols solutions, the surface of GC working electrode was first mechanically polished by 0.05 μm alumina slurry and then thoroughly rinsed with Milli-Q water. After mechanical polishing, the GC electrode was cleaned electrochemically by cyclic voltammetry (in the potential range from -0.2 to 1.0 V, using a scan rate of 50 mV s^{-1}) in supporting electrolyte (0.1 mol L^{-1} acetate buffer solution of pH 3.6), until the steady-state cyclic voltammograms were obtained. This pretreatment procedure of GC electrode ensured very reproducible CV, DPV and SWV measurements in solutions of investigated flavonols.

2.3. Data collection and analysis

All voltammograms (CVs, DPVs, and SWVs) were collected and analyzed using electrochemical software Model 270/250 (EG&G Princeton Applied Research) to get the electrochemical results (peak potentials, peak currents, peak width at the half height of anodic peaks). The drawing of all Figures from the experimental measurements have been done by OriginPro 2015 software (OriginLab Corporation, Northampton, USA). Chemical structures have been drawn using ChemDraw® Professional 15.0 Software (Perkin Elmer Informatics, USA).

3. RESULTS AND DISCUSSION

Electrochemical behaviour of quercetin, morin, and rutin, the concentration of $3 \cdot 10^{-5}$ mol L^{-1} was investigated in 0.1 mol L^{-1} sodium acetate-acetic acid buffer solution of pH 3.6, using cyclic voltammetry (CV), differential pulse voltammetry (DPV), and square-wave voltammetry (SWV) methods.

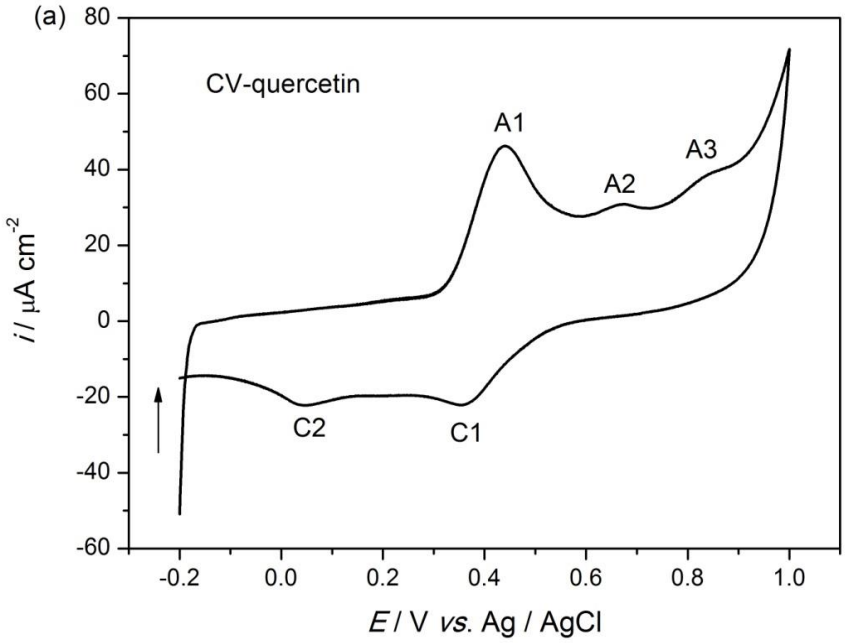
3.1. Electrochemical behaviour of quercetin

Quercetin (3,5,7,3',4'-pentahydroxyflavone) has been constituted by two benzene rings (catechol B ring and resorcinol A ring) joined by a heterocyclic C ring, with five -OH groups attached to A, B, and C rings (see Scheme 1.). Such structure, especially coupling between B ring and C ring through the 2,3-double bond, gives quercetin molecules excellent electron-donating properties (much better then have flavanols with similar chemical structure, e.g. catechin). Also, deprotonation of -OH groups (dissociation) was facilitated due to the charge delocalization. Quercetin is a weak polyprotic acid, and depending on the pH of the solution may exist un-dissociated or in many anionic forms. Three to five dissociation constants were reported in the literature for quercetin. Thus, Jovanović et al. [39] reported values of pK_{a1}= 6.74; pK_{a2}= 9.02; pK_{a3}=11.55, Herrero-Martinez et al, [40] reported values of pK_{a1}= 7.59; pK_{a2}= 9.33; pK_{a3}=11.56, while Momić et al. [41] reported five dissociation constants for quercetin: pK_{a1}=5.50, pK_{a2}=7.15, pK_{a3}=8.00, pK_{a4}=9.57 and pK_{a5}=11.40. Dissociation diagram of quercetin, calculated from their dissociation constants using CurTiPot software [42] was not presented here, but the diagram shows that at pH 3.6 (pH in our investigations) only fully protonated neutral quercetin molecules exists in solution. Therefore neutral quercetin species participate in all electrochemical oxidation reactions of quercetin performed in this study.

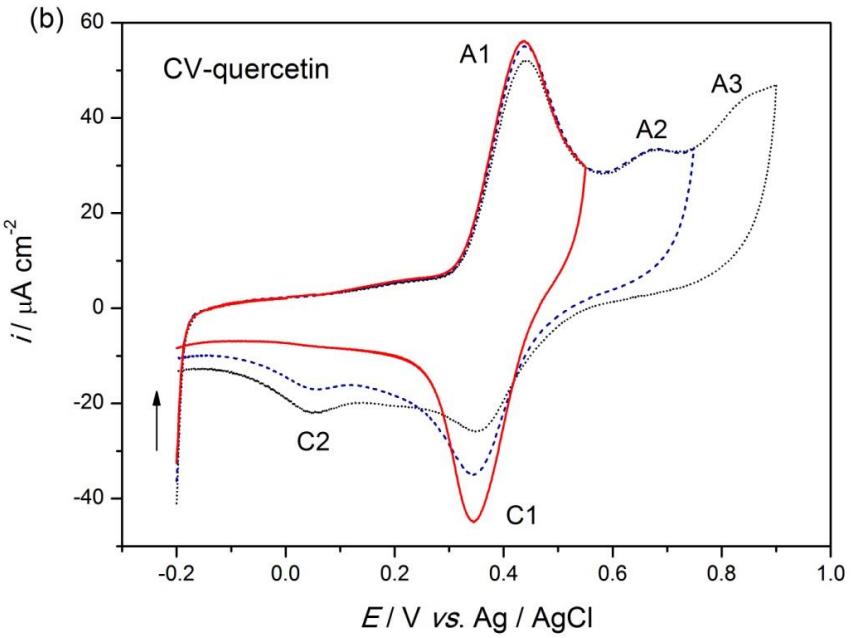
Quercetin has five -OH groups than can be oxidized (Scheme 1.). It was reported earlier by Jovanović et al., [43] that quercetin has favourable electron-donating properties in comparison to many other flavonoids, which originated from the electron donating 3-OH group in the C ring conjugated through a 2,3-double bond to the 3',4'-catechol structure of B ring. According to this report catechol B ring in the structure of quercetin was significantly easily oxidized than resorcinol A ring. Such conclusion was also confirmed by quantum chemical calculations studies of reactivity of -OH groups in quercetin structure, i.e. their bond dissociation enthalpy, BDE [44-45]. Authors were shown by the

BDE values that H-transfer from B ring (4'-OH and 3'-OH groups) is much easier (due to the lower BDE values), than from A ring (7-OH and 5-OH groups) or ring C (3-OH group).

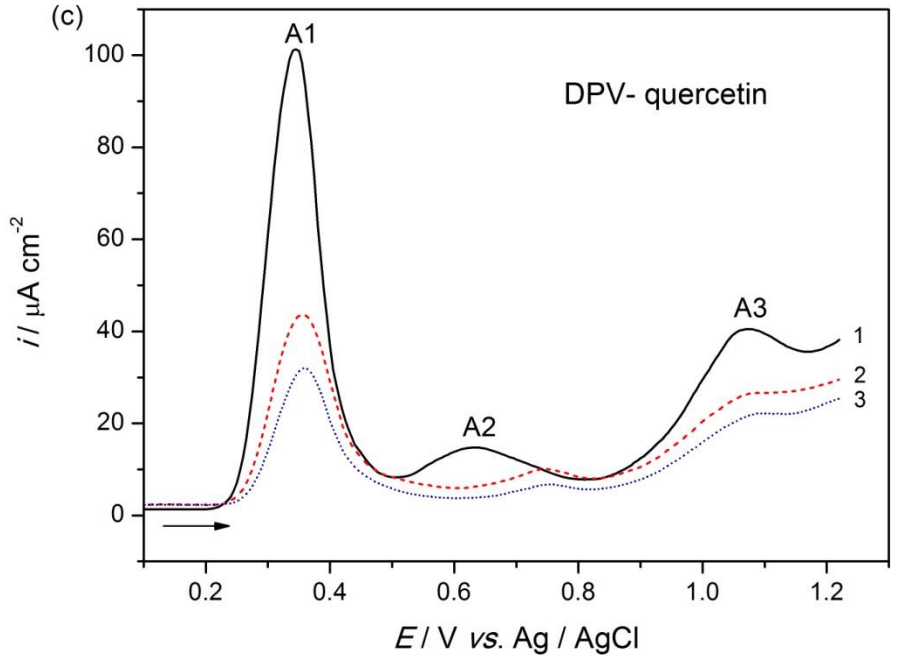
Cyclic voltammetry (CV) was used first to study the electrochemical behaviour of quercetin. Cyclic voltammograms (CVs) of quercetin show three anodic oxidation peaks, peak A1 at 0.440 V, peak A2 at 0.676 V and peak A3 at 0.830 V (Figs. 1.a, 1.b., and Table 1.).



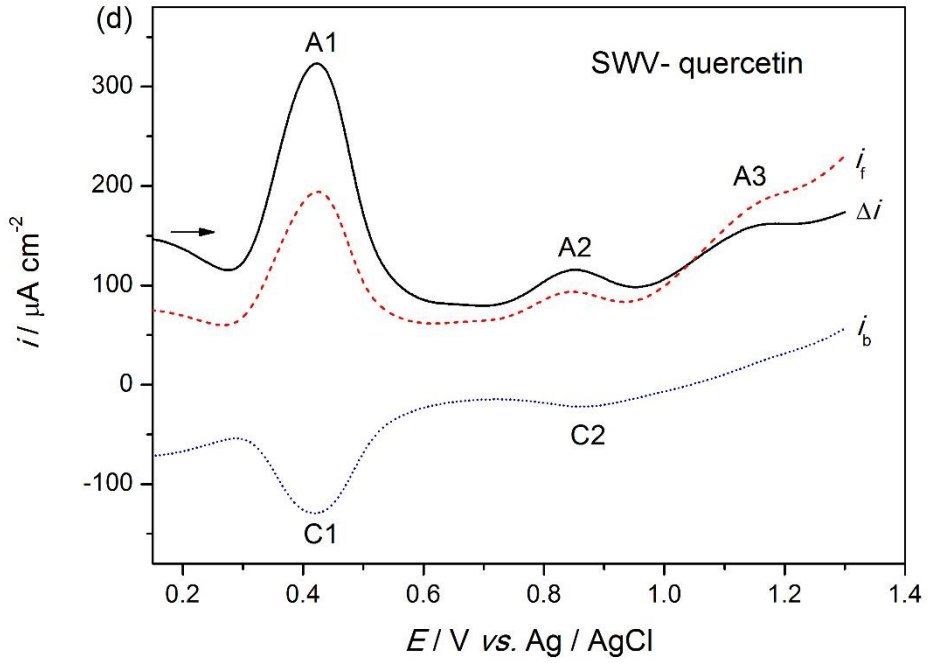
A



B



C



D

Figure 1. Cyclic voltammograms (Figure 1a, 1b), differential pulse voltammograms (Figure 1c), and square-wave voltammograms (Figure 1d) of quercetin, c=3·10^{-5} mol L^{-1}, in acetate buffer solution pH 3.6. The scan rate of CV 100 mV s^{-1}. The experimental conditions for DPV and SWV measurements were given in section Experimental.

Table 1. Electrochemical parameters of quercetin, morin, and rutin obtained from CV, SWV, and DPV measurements

Flavonol	Peak	CV $E_{p,a}$ / V	CV $E_{p,c}$ / V	SWV $E_{p,a}$ / V	SWV $E_{p,c}$ / V	DPV $E_{p,a}$ / V
Quercetin	1	0.440	0.354	0.422	0.420	0.345
	2	0.676	0.047	0.852	0.862	0.635
	3	0.830	-	1.150	-	1.075
Morin	1	0.462	0.374	0.470	0.476	0.390
	2	-	-	-	-	-
	3	1.060	-	1.158	-	1.015
Rutin	1	0.477	0.421	0.486	0.492	0.430
	2	-	-	-	-	-
	3	1.150	-	1.132	-	1.095

Although the pathways of electrochemical oxidation of quercetin were extensively investigated, some details of these processes are still not completely understood. Generally, in the literature was accepted that the first anodic oxidation peak A1 corresponds to the reversible (or quasi-reversible) oxidation of catechol 3'-OH and 4'-OH groups on the B ring to 3',4'-quinone structure [9-23]. This oxidation reaction is pH dependent and proceeds by a two-electron ($2e^-$)-two-proton ($2H^+$) oxidation reaction mechanism [11-13, 16, 22]. The cathodic peak C1 was observed at 0.354 V (see Figs. 1a, 1b. Table 1) and corresponds to the reduction of the 3',4'-quinone formed in the first oxidation peak A1, back to the catechol structure. The peak separation of the first redox peak couple, $\Delta E_p = E_{p,a} - E_{p,c} = 86$ mV, is higher than the theoretical value of 30 mV for two electron process, what could point to a slow electron-transfer reaction on GC electrode or contribution of some chemical reaction at GC electrode after the charge transfer reaction. The higher peak separation value of the first anodic oxidation/reduction peak of quercetin was also reported by some other authors [10, 12, 16, 22]. Additional CV experiments at various scan rates ranging from 25 to 150 mV s^{-1} were conducted (not presented here). These CVs shows that the mechanism and reversibility of the oxidation reaction corresponding to peak A1 were highly scan-rate dependent. The current of peak A1 changes linearly with the square-root of the scan rate. These results give an indication that the initial stage of quercetin electrochemical oxidation is under diffusion control. This observation was in agreement with results reported by other authors [7, 10, 12, 20]. Also, we found that if the scan rate increase the reversibility of this oxidation process increase. That was demonstrated by the increase of peak current ratio ($i_{p,c}/i_{p,a}$) of peak C1 and A1 with an increase in the scan rate. Also, the scan-rate normalized anodic current of peak A1 ($i_{p,a}/v^{1/2}$) *vs*. scan rate (v) decreases with increasing the scan rate. Such results indicate an electrochemical-chemical (EC) mechanism, i.e. chemical rearrangement of oxidation product(s) formed after the first quercetin oxidation step (peak A1). The less time for the rearrangement reactions (i.e., the faster the scan rate) means that more oxidation products are available to be reduced (i.e. higher cathodic peak current). The EC oxidation mechanism of quercetin was also reported in the papers of Hendrikson et al. [9], Zare et al. [12], Timbola et al. [13], and Kummer et al. [21].

The assignation of the second oxidation peak of quercetin A2 at 0.676 V (Figs.1a, 1b.) is not so clear in the published papers. Most of the authors [9, 11, 14-15, 17, 22-23] ascribed the second oxidation peak of quercetin to the irreversible oxidation of 3-OH group in the C ring of quercetin (see Scheme 1). However, Timbola et al. [13] ascribed peak A2 to the oxidation of chemical by-products formed in the preceding electrochemical step at peak A1. Our results suggest that peak A2 could be assigned to the oxidation of -OH group at the C-3 position in the structure of quercetin (see also an explanation of results for rutin). Also, our results suggest that this oxidation process is reversible and not irreversible as was reported by other authors, because reduction peak C2 at 0.047 V was clearly observed when polarization was reversed after the peak A2 (see Fig. 1b.). According to our best knowledge in our investigation for the first time was observed that second reduction peak C2 of quercetin corresponds to 3-OH group on ring C.

The third anodic oxidation A3 was observed at a potential of 0.830 V. This peak could be ascribed to oxidation of hydroxyl groups at position 5 and 7 in ring A (see Scheme 1.). Such assignation was also reported by other authors [9, 11, 14-15, 17]. However, Timbola et al. [13] ascribed peak A3 (as also peak A2) to the oxidation of chemical by-products formed in the preceding electrochemical steps. Our results suggest that process of electrochemical oxidation of 5,7-OH groups in A ring is an irreversible process because reduction peak corresponded to this oxidation was not observed during reverse CV scan (see Fig.1b). Such conclusion was also reported by Hendrikson et al. [9], Zielinska and Pierozynski [15], and Makhotkina and Kilmartin [17].

Differential pulse voltammetry (DPV) measurements confirmed the presence of three oxidation peaks of quercetin (Fig. 1.c). Anodic oxidation peaks A1, A2 and especially A3, were much better expressed than in CV, probably due to the higher sensitivity of DPV method. The oxidation potential of peak A1 obtained in DPV measurements has a lower value (see Table 1.) than in CV measurements, probably due to the lower scan rate of DPV (5 mV/s in DPV in comparison to CV where scan rate was 100 mV/s). The results of DPV confirmed the mechanism of quercetin oxidation proposed by CV measurements. The strong adsorption of quercetin and its oxidation product(s) which blocked the electrode surface was observed since the current of oxidation peaks decreased drastically during the successive anodic scans (Fig 1.c).

Square-wave voltammetry (SWV) was also used for investigation of the electrochemical behaviour of quercetin. SW voltammograms of quercetin (Fig. 1.d) confirmed the results of CV and DPV measurements, i.e. the existence of three anodic oxidation peaks A1, A2 and A3.

The reversibility of peaks A1 and A2 was clearly shown (oxidation and reduction peaks occur at practically the same potential values, see Fig. 1d and Table 1.). According to our best knowledge, the results of our SWV and CV measurements are the first evidence that the second oxidation reaction of quercetin corresponded to oxidation of 3-OH group at C ring shows reversibility (peaks A2 and C2). As in DPV measurements, strong adsorption of quercetin and its oxidation product(s) was observed, and the current of peaks A1, A2, and A3 significantly decreased during the second and third successive SWV scans (diagram not shown here).

3.2. Electrochemical behaviour of morin

Morin (3,5,7, 2',4'-pentahydroxyflavone) has a structure similar to quercetin but differ in the position of -OH groups on B ring. Morin has two -OH groups at position 2',4' (resorcinol structure) in contrast to quercetin with 3',4' -OH groups (catechol structure) on B ring (see Scheme 1.). Such difference in position of -OH groups on ring B influence on the different electrochemical behaviour of morin in comparison to that of quercetin. Also, the different position of -OH groups influence on the values of dissociation constants. Two or three dissociation constants were reported in the literature for morin. Thus, Jovanović et al. [39] reported values of pK_{a1}= 3.46; pK_{a2}= 8.10, while Herrero et al. [40] reported three dissociation constants for morin: pK_{a1}=4.99, pK_{a2}=8.29, and pK_{a3}=10.33. Dissociation diagram of morin, calculated from their dissociation constants (not shown here), show that at pH 3.6 mostly fully protonated neutral morin molecules exist and therefore these species participate in electrochemical oxidation reactions of morin performed in this study.

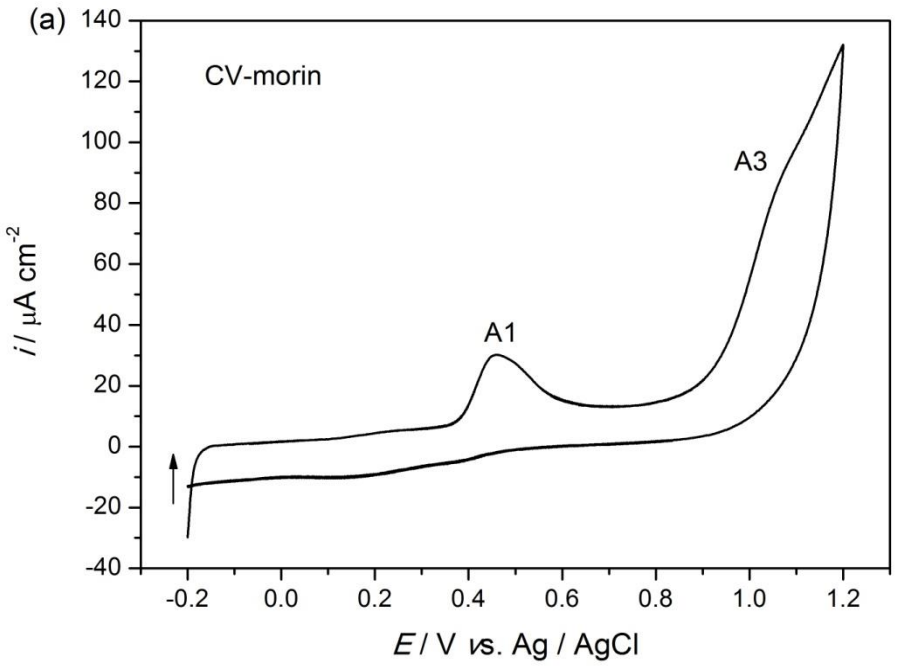
Morin (as also quercetin) has five -OH groups than can be oxidized (see Scheme 1.). However, the difference in position of -OH groups in B ring, strongly influences on electron-donating properties and oxidation ability of morin in comparison to quercetin, as was reported in the paper of Jovanovic et al. [43] where they investigated the importance of *ortho*-diphenyl arrangement in the ring B on reduction potential of flavonoids.

The electrochemical behaviour of morin is still not-very-well understood, and the published results are very different in many conclusions. Thus, Janeiro and Brett [24] reported that this first oxidation peak corresponds to the oxidation of the 2',4'-dihydroxy moiety at B ring and involved one-electron-one-proton reversible reaction. Oxidation was pH depended and proceeds through EC reaction mechanism. The second oxidation peak should be associated with irreversible oxidation of 5,7-dihydroxyl moiety of ring A by one-electron-one proton process. Kang et al. [25] reported only one anodic oxidation peak of morin and ascribed this peak to irreversible two-electron-two-proton oxidation of 2',4'-OH groups to 2',4'-quinone structure. Ping et al. [26] and Wang et al. [27] also reported only one anodic oxidation peak of morin involving the loss of two electrons and two protons. According to their results, this reaction is totally irreversible and was not assigned to any specific -OH groups in the structure of morin. He et al. [28] reported two oxidation peaks on morin. They claimed that 4'-OH group was first oxidized at lower potential forming phenoxyl radicals *via* a one-electron-one-proton reaction and after at higher anodic potential 2'-OH group of morin was oxidized by the same mechanisms. Both oxidation reactions they observed to be reversible. Masek et al. [29] reported that the oxidation of 2',4'-dihydroxy moiety at the B ring of morin occurs first, at relatively low positive anodic potentials, and is one-electron-one-proton irreversible reaction. The hydroxyl group at position 3 on ring C should be oxidized afterward, also by the one-electron-one-proton irreversible process. Ziyatdinova et al. [30] reported that morin is irreversibly oxidized showing two well-defined irreversible oxidation peaks. The first oxidation peak corresponds to the irreversible oxidation of 2',4,'-dihydroxy moiety at B ring, while the second oxidation peak was associated with the irreversible oxidation of hydroxyl group at position 3 on ring C. Each of these two oxidation processes involves one-electron and one-proton exchange. Kummer et al. [20] studied electrochemistry of morin by CV and DPV and found that oxidation of morin is an irreversible process with one anodic peak. This peak

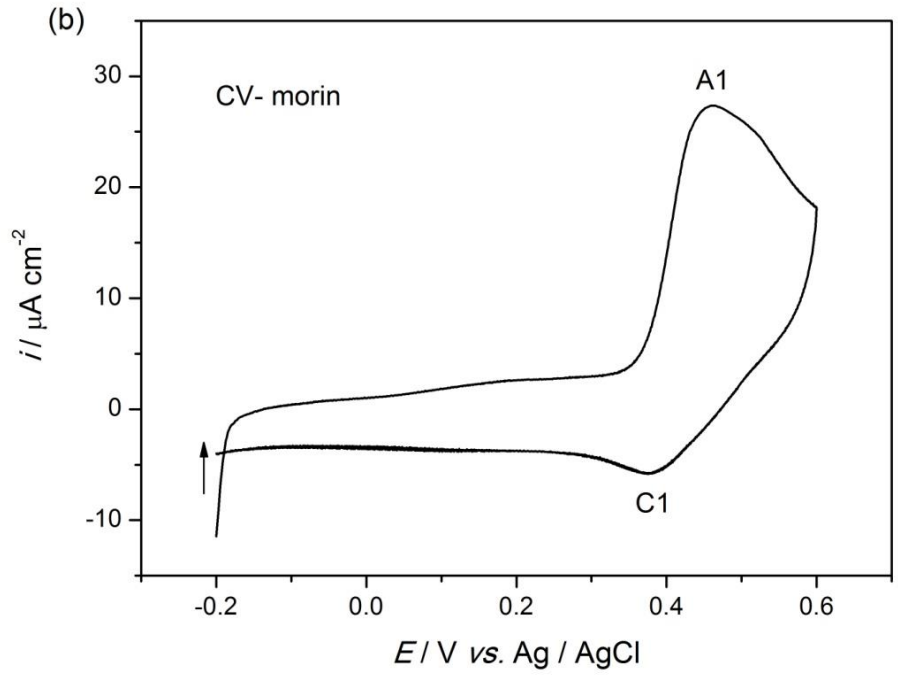
they ascribed to the quasi-reversible one-electron-one-proton oxidation of 2',4'-dihydroxy moiety at ring B. They also performed the quantum chemical calculation of proton affinities (PA), electron transfer enthalpies (ETE) and sequential proton loss electron transfer (SPLET) values of 2'-OH, and 4'-OH groups on the B ring of morin. The values of these chemical parameters (ETE and SPLET values) can indicate the possible reactive sites and radical species formed during electrochemical oxidation of morin. Their electrochemical measurements and quantum chemical calculations can not give definitive conclusion which of these to groups on B ring were oxidized to the phenoxy radical form, because 2'-OH and 4'-OH group shows practically the same PA, ETE, and SPLET values. In the second paper of this subject, Kummer et al. [21] used the combination of electrochemical flow cell coupled online with electrospray ionisation-mass spectrometry (ESI-MS) for identification of the short-lived products generated during electrochemical oxidation of morin. Different oxidation products which are in tautomeric equilibrium were observed by ESI-MS. First oxidation step corresponds to the formation of 4'-phenoxy radical by the one-electron-one-proton mechanism. This radical is in equilibrium with 3-phenoxy radical form. During the second electrochemical step by one-electron-one-proton mechanism, 4',3-phenoxy di-radical was formed. This oxidation product can be transferred to the 2',4'-*meta*-quinone or 4',3-quinone structure. During further chemical intramolecular rearrangements or addition reactions with nucleophiles (e.g. methanol used as a solvent in their experiments) different adduct products were formed.

Therefore based on all investigations mentioned above, it could be concluded that the number and assignation of oxidation and reduction peaks of morin and in particular the mechanism of the electrochemical oxidation of morin is still not fully understood. Therefore to get some additional information, we investigated some of the aspects of the electrochemical behaviour of morin by CV, DPV, and SWV methods.

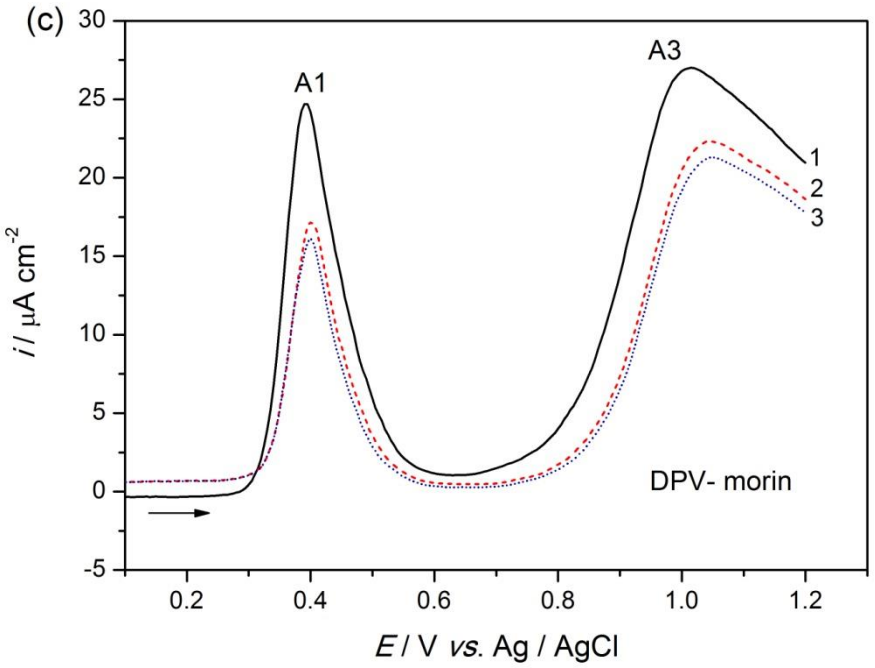
Our cyclic voltammetry measurements of morin show the presence of two anodic oxidation peaks, peak A1 at 0.462 V and peak A3 at 1.060 V (Figs. 2.a, 2.b. and Table 1.). According to CV results (Figs. 2a. and 2b., and also SWVs, Fig. 2d.), it seems that the first oxidation peak A1 corresponded to reversible oxidation of 2',4'-OH groups on B ring. The small reduction peak C1 was observed at 0.374 V (see Fig. 2b. Table 1) and corresponds to the reduction of the oxidation products formed in peak A1. The peak separation $\Delta E_p = E_{p,a} - E_{p,c} = 88$ mV is relatively close to the theoretical value of 60 mV for one-electron process. Therefore, the process corresponds to peak A1 could be a reversible one-electron-one-proton process of oxidation of morin to a phenoxy radical, as was also reported by Janeiro and Brett [24]. However, is still not-fully-understood which of these to groups on B ring were oxidized to the phenoxy radical form. Such conclusion is because 2'-OH and 4' OH groups show very similar quantum chemical parameters values, e.g. values of proton affinities (PA), electron transfer enthalpies (ETE) and sequential proton loss electron transfer (SPLET) values [20] and bond dissociation enthalpy BDE [46].


(a)
CV-morin
A3
A1
i / μA cm^{-2}
E / V *vs*. Ag / AgCl

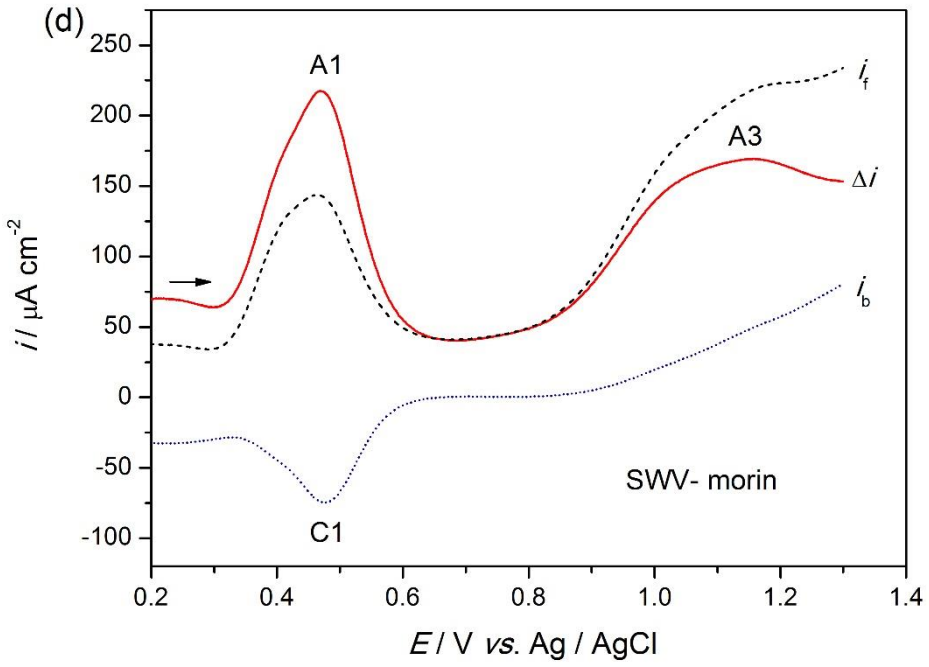
A


(b)
CV- morin
A1
C1
i / μA cm^{-2}
E / V *vs.* Ag / AgCl

B



C



D

Figure 2. Cyclic voltammograms (Figure 2a, 2b), differential pulse voltammograms (Figure 2.c), and square-wave voltammograms (Figure 2d) of morin, c=3·10^{-5} mol L^{-1}, in acetate buffer solution pH 3.6. The scan rate of CV 100 mV s^{-1}. The experimental conditions for DPV and SWV measurements were given in section Experimental.

Also, it was observed that the reduction peak C1 was disappeared at scan rates lower than 100 mV s^{-1}. This scan rate-dependence could indicate an electrochemical-chemical (EC) reaction mechanism, i.e. chemical rearrangement following the electrochemical oxidation reaction. If the less time is allowed for the rearrangement reaction (i.e., the faster scan rate was used), the more oxidation product is available to be reduced (as in the case of quercetin, but at morin, this process is more expressed). Janeiro and Brett [24] and Hendrikson et al. [9] also proposed such explanation. The EC mechanism of oxidation of morin was also observed by the combination of electrochemical flow cell coupled online with electrospray ionisation-mass spectrometry (ESI-MS), where different oxidation products which are in tautomeric equilibrium were observed [21].

The oxidation peak of morin A3 at 1.060 V (Figs.2a, 2b.) could be assigned to the oxidation of 5,7- dihydroxyl moiety on ring A (see an also explanation for rutin), This oxidation is an irreversible process, because no reduction peak was observed. The absence of oxidation peak A2 of morin, i.e. oxidation of -OH group at position 3 at ring C could be explained possibly by the formation of some intermolecular hydrogen bond with the oxygen at position 4 at ring C.

DPV measurements also show the presence of two oxidation peaks of morin, A1, and A3 (Fig. 2.c). Anodic oxidation peaks A1 and A3 are very high in comparison to that observed by CV, probably due to the higher sensitivity of DPV. The value of oxidation peak A1 obtained in DPV measurements has significantly lower value (see Table 1.) than those in CV measurements, probably due to the lower scan rate of DPV. The results of DPV confirmed the mechanism of morin oxidation proposed by CV. Morin and its oxidation products are strongly adsorbed on the electrode surface since the current of oxidation peaks decreased drastically during the successive anodic scans (Fig 2.c).

Similar results to CV and DPV measurements were also observed by SWV (Fig. 2.d), confirmed the existence of two oxidation peaks A1 and A3. The reversibility of peak A1 was clearly shown because oxidation and reduction peak occur at practically the same potential value (see Fig. 2d and Table 1.). Also, irreversibility of peak A3 observed in CV measurements was clearly shown by SWV. As in DPV measurements, strong adsorption of morin and its oxidation product(s) drastically decrease the current of peak A1 during the successive positive SWV scans (diagram not was shown).

3.3. Electrochemical behaviour of rutin

Rutin (3',4',5,7-tetrahydroxyflavone-3β-D-rutinoside; quercetin-3-*O*-rutinoside) has a chemical structure very similar to quercetin, the difference is only at position 3 on C ring. Rutin is a glycoside of quercetin with a disaccharide rutinose bonded at position 3 on C ring (see Scheme 1). The difference in the structure of ring C influenced on values of dissociation constants of rutin in comparison to that of quercetin. Three or four dissociation constants were reported in the literature for rutin. Thus, Jovanović et al. [39] reported values of pK_{a1}= 7.10; pK_{a2}= 9.15, and pK_{a3}=11.65, while Mielczarek [47] reported four dissociation constants for rutin: pK_{a1}=7.35, pK_{a2}=8.80, and pK_{a3}=11.04 and pK_a=11.90. Dissociation diagram of rutin, calculated from their dissociation constants, show that neutral rutin species participate in oxidation reactions of rutin at pH 3.6.

Rutin has four -OH groups which can be oxidized (one less than quercetin, see Scheme 1.). Such difference in the number of -OH groups and rutinose bonded at position 3 on ring C, must strongly influence on electron-donating properties and oxidation ability of rutin in comparison to that of quercetin. A similar result was reported in the study of Jovanovic et al. [43], where they investigated the importance of 3-OH group in the ring C on reduction potential of flavonoids.

The electrochemical behaviour of rutin is still not fully understood, and the published results are sometimes different in conclusions. Thus, Ghica and Brett [31] reported that catechol 3',4'-dihydroxyl group of rutin (B ring) was first oxidized by a two-electron-two-proton reversible oxidation reaction, followed by an irreversible oxidation reaction of 5,7-dihydroxyl group (A ring). Both reactions are pH dependent. Tian et al. [32] reported that rutin exhibits one well-defined reversible peak couple and one irreversible oxidation peak. The first reversible peak couple has been assigned to the oxidation of dihydroxyl group at B ring of rutin, and the second irreversible anodic peak was attributed to the oxidation of the -OH groups at A ring. The first electrode reactions are adsorption controlled, with the transfer of two-electrons and two-protons. The electrochemical behaviour was found to be pH dependent. Zare et al. [33] investigated the electrochemical behaviour of rutin on inactivated, activated, and multi-wall carbon nanotubes modified GCE by CV and chronocoulometry. At inactivated GCE rutin shows the redox pair with ΔE_p=76 mV. The anodic and cathodic peak currents increase linearly with the square root of the scan rate, suggesting that the reaction is mass-transport controlled. The peak current ratio ($I_{p,c}/I_{p,a}$) increases gradually with the increase of the potential scan rate. Also, the normalized current ($I_{p,a}/v^{1/2}$) diminishes gradually with increase in the scan rate. These results are characteristics of an EC mechanism. The subsequent chemical reaction (after charge transfer) can be considered as a dimerization, hydroxylation, or intramolecular reaction. Liu et al. [34] investigated electrochemistry of rutin on MWCNTs-IL-Gel-GC electrode. They reported quasi-reversible two-electron-two-proton process forming 3',4'-quinone. With the scan rate increasing, the reaction mechanism varied from surface controlled to diffusion controlled. Oliveira-Roberth et al. [36] investigated rutin by CV and DPV at Screen Printed carbon electrode (SPE). They found that the first redox pair was attributed to the catechol moiety (3',4'-dihydroxy groups on the B ring) which undergoes a quasi-reversible oxidation process (ΔE_p was approximately 200 mV, and $I_{p,c}/I_{p,a}$ <1) leading to the corresponding *o*-quinone structure. In turn, the second oxidation peak at higher potentials was attributed to the irreversible electrochemical oxidation of resorcinol moiety of ring A to phenoxy radical form, which was followed by a chemical process of dimerization (EC mechanism). The electrochemical oxidation of rutin was followed by a strong adsorption of oxidation products in both oxidation processes. The process is pH dependent. Pinar et al. [37] investigated electrochemistry of rutin by CV and SWAdSV on the boron-doped diamond electrode (BDDE). CV showed a pair of redox peaks at lower potentials corresponds to the oxidation of catechol moiety (3',4'-dihydroxy groups) on the B ring of rutin and the reduction of the 3',4'-quinone product, respectively. The second oxidation peak was assigned to the irreversible electrochemical oxidation of resorcinol moiety (5',7'-dihydroxy groups) on the A ring. The difference of peak potentials of the first redox pair is 93 mV. The electrode reaction is controlled by the adsorption. Subsequent CV scans result in a decrease of current due to adsorption of oxidation products. Yang et al. [38] investigated electrochemistry of rutin by CV and DPV on graphene nanosheets modified glassy carbon electrode (GS/GCE). They found that

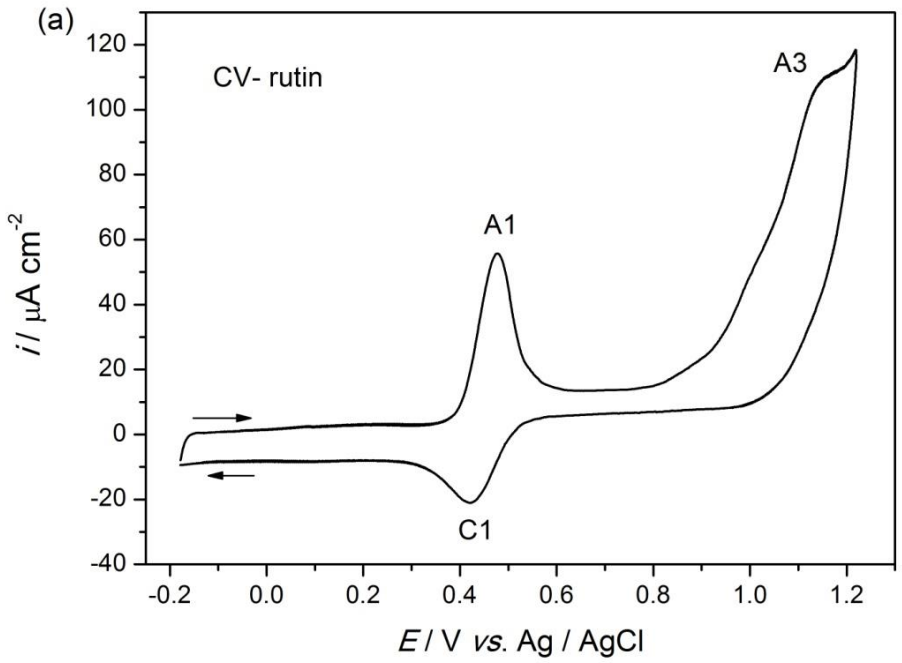
oxidation of rutin was a reversible two-electron two-proton process, with a ΔE_p separation of about 27 mV.

By papers mentioned above it could be concluded that some aspects of electrochemistry of rutin are still not fully understood. Therefore, we investigated the electrochemical behaviour of rutin by CV, DPV and SWV methods to get some additional information regarding this subject.

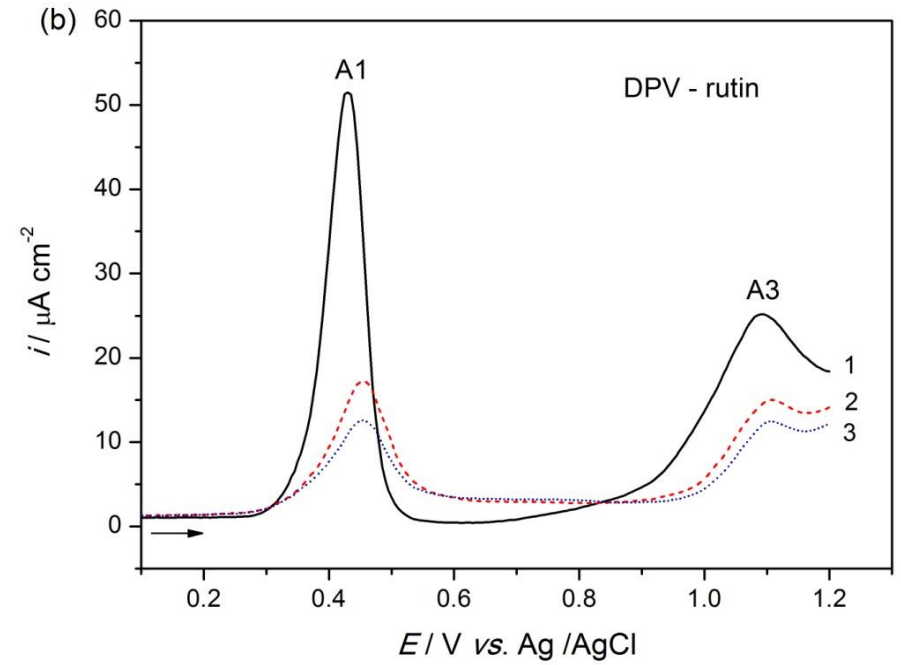
Our cyclic voltammetry of rutin showed two anodic oxidation peaks, peak A1 at 0.477 V and peak A3 at 1.150 V (Fig. 3.a. and Table 1.). The first redox couple (peaks A1 and C1) corresponds to the oxidation of 3',4'-dihydroxy substituent on the B ring of rutin and the reduction of the 3',4'-quinone, respectively [31-32, 34, 36-37]. This reaction is reversible (or quasi-reversible), pH depended and proceeded through EC reaction mechanism [9, 33].

The peak separation $\Delta E_p = E_{p,a} - E_{p,c} = 56$ mV is higher than the theoretical value of 30 mV for two-electron process. Also, it was observed that ΔE_p increase with increasing scan rates indicating that oxidation process appears more as quasi-reversible. The peak current ratio ($I_{p,c}/I_{p,a}$) increases gradually with the increase of the potential scan rate, what is characteristics of an EC mechanism. The subsequent chemical reaction can be considered as a dimerization, hydroxylation or intramolecular reaction [33].

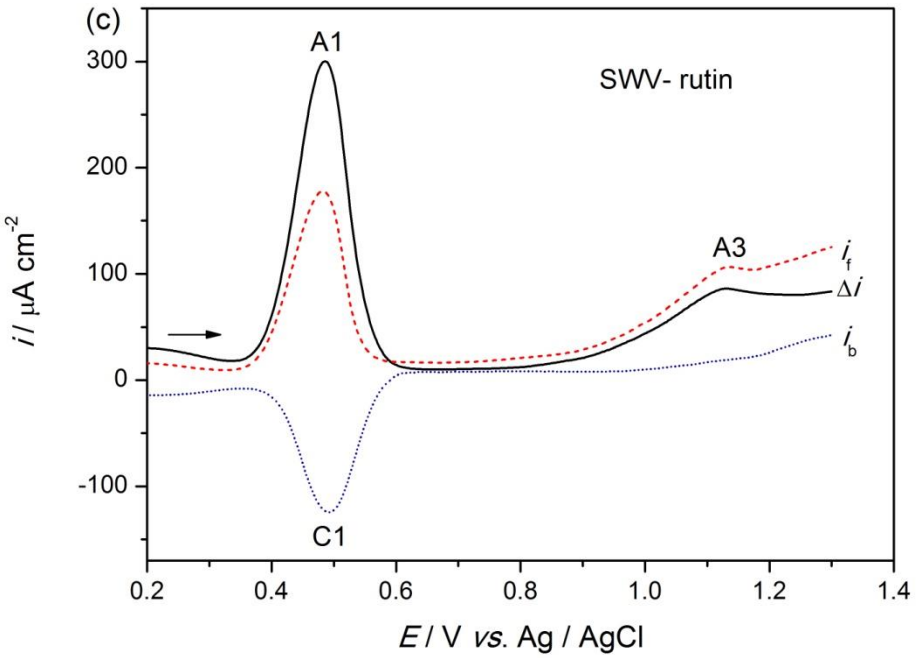
The oxidation peak of rutin A3 at 1.150 V (Figs.3a.) should be associated with oxidation of 5,7- dihydroxy moiety of ring A [31-32, 36-37], This oxidation is an irreversible process because no reduction peak was observed. The absence of oxidation peak A2 could be explained by the presence of rutinoside group without electroactivity at position 3 of C ring of rutin.



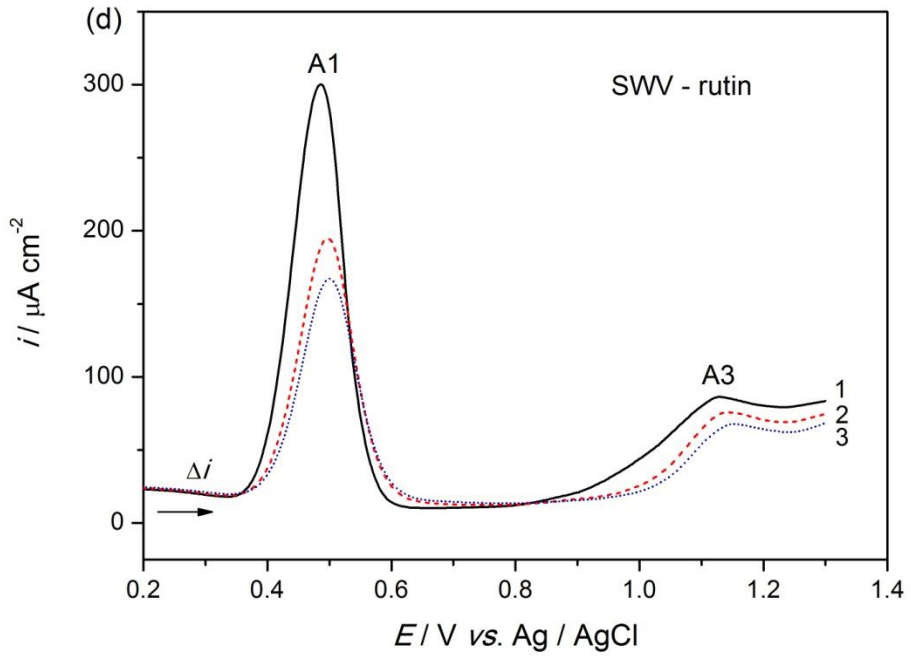
A


(b)
A1
DPV - rutin
A3
1
2
3
i / μA cm^{-2}
E / V *vs*. Ag /AgCl

B


(c)
A1
SWV- rutin
A3
i_f
Δ*i*
i_b
C1
i / μA cm^{-2}
E / V *vs*. Ag / AgCl

C



D

Figure 3. Cyclic voltammogram (Figure 3a), differential pulse voltammograms (Figure 3b), and square-wave voltammograms (Figure 3c, 3d) of rutin, c=3·10^{-5} mol L^{-1}, in acetate buffer solution pH 3.6. The scan rate of CV 100 mV s^{-1}. The experimental conditions for DPV and SWV measurements were given in section Experimental.

DPV measurements also show the presence of two oxidation peaks, A1 and A3 (Fig.3.b). Anodic oxidation peaks A1 and A3 are much higher in comparison to CVs, probably due to the higher sensitivity of DPV. The value of oxidation peak A1 obtained in DPV measurements has significantly lower value (see Table 1.) than those in CV measurements, probably due to the lower scan rate of DPV. The results of DPV confirmed the mechanism of rutin oxidation proposed by CV. Rutin and its oxidation products are strongly adsorbed on the electrode surface since the current of oxidation peaks decreased drastically during the successive anodic scans (Fig 3.b). Such behaviour was also observed by Ghica and Brett [31].

SW voltammograms (Fig. 3.c) confirmed the existence of two oxidation peaks A1 and A3. The reversibility of peak A1 was clearly shown because oxidation and reduction peak occur at practically the same potential value (see Fig. 3c and Table 1.). Also, irreversibility of peak A3 observed in CV measurements was clearly shown by SWV. As in DPV measurements, strong adsorption of rutin and its oxidation product(s) decrease the current of both peaks especially that of peak A1 during the successive SWV scans (Fig. 3 d.).

4. CONCLUSIONS

The electrochemical behaviour of three flavonols, quercetin, morin, and rutin was investigated by CV, DPV, and SWV methods. The study has shown that electrochemical behaviour of investigated flavonols strongly depends on their chemical structure, particularly on the number and position of electron-donating -OH groups on rings A, B, and C. The important factors of the electrochemical oxidation of flavonols are as follows. (i) The presence of *ortho*-3',4',-dihydroxy groups (catechol group) on the B ring. (ii) The conjugation of the B ring with the C ring through 2,3-double bond. (iii) The presence of electron-donating 3-OH group and 4-oxo group in the structure of C ring. (iv) The electron-donating 5-OH and 7-OH groups on A ring also contributes to the electrochemical activity of investigated flavonols.

Quercetin satisfies all of the requirements mentioned above and therefore shows the best electron-donating properties and lower oxidation potential of the first oxidation peak (A1) in comparison to that of morin and rutin. The peak A1 corresponds to the reversible oxidation of 3',4',-OH groups (catechol moiety) on B ring to the *ortho*-quinone structure by two-electron-two-proton process. This electrochemically active and unstable *ortho*-quinone species then undergoes chemical rearrangements or addition reactions. This suggests an electrochemical-chemical (EC) reaction mechanism. At higher anodic potential 3-OH group on ring C was oxidized (peak A2), by the reversible one-electron-one-proton reaction. According to our best knowledge in our investigation was for the first time observed that second reduction peak C2 of quercetin corresponds to 3-OH group on ring C. The 5-OH and 7-OH groups on ring A were oxidized at higher anodic potentials (peak A3). This oxidation is an irreversible process.

Morin with *met*a-2',4', dihydroxyl groups (resorcinol moiety) shows the higher value of oxidation potential of peak A1 than quercetin, indicating that oxidation of 2',4'-OH groups on B ring to quinone structure is more difficult in comparison to quercetin. This fact clearly indicates the importance of the adjacent of two hydroxyl groups in the *ortho*-diphenolic arrangement in the ring B of flavonols. The first oxidation process (peak A1) is one-electron-one-proton reversible reaction, proceeds through an EC mechanism. The oxidation peak of morin A3 should be associated with irreversible oxidation of 5,7- dihydroxyl moiety on ring A. The absence of oxidation peak A2, i.e. oxidation of 3-OH group on ring C, could be explained possibly by the formation of some intermolecular hydrogen bond between the 3-OH group and oxygen at position 4 on ring C.

Rutin (quercetin-3-*O*- rutinose) shows the highest oxidation potential of peak A1. The glycosylation with rutinose on position 3 in the C ring blocked the oxidation process at this position, and decreases the strength of delocalization of electrons from B ring. Therefore, the oxidation of B ring of rutin was observed at significantly higher potentials than that of quercetin. This result shows how important is the role of 3-OH group on the C ring on the electrochemical properties of flavonols. The first oxidation peak A1 corresponds to the oxidation of 3',4'-dihydroxy substituent on the B ring of rutin. This reaction is reversible (or quasi-reversible), pH-dependent process and proceeds through EC reaction mechanism. The oxidation peak of rutin A3 should be associated with the irreversible oxidation of 5,7-dihydroxyl moiety on ring A. The absence of oxidation peak A2 could be explained by the presence of rutinoside group without electrochemical activity, at position 3 on C ring of rutin.

The results observed in this investigation are in agreement with the results of some other authors which investigate the electrochemistry of quercetin, morin, and rutin. At the same time, we observed some new informations regarding the electrochemical oxidation/reduction processes of these flavonols, such as follows. (i) For the first time, we observed the presence of cathodic reduction peak C2 of quercetin which corresponds to the 3-OH group at C ring. (ii) The reversibility of the first oxidation process of morin (peaks A1 and C1) was clearly demonstrated. (iii) It was clearly shown how important is the role of -OH groups (i.e. their number and their position on rings A, B, and C) on the electrochemical behaviour of investigated flavonols.

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