Effect of 3-O-Galloyl Substitution on the Electrochemical Oxidation of Quercetin and Silybin Galloyl Esters at Glassy Carbon Electrode

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Abstract
The galloyl substitution effect on the antioxidant potential of quercetin-3-O-gallate (QG) and silybin-3-O-gallate (SBG), and the oxidation of QG and SBG were studied by cyclic, differential, and square-wave voltammetry using a glassy carbon electrode, and compared with their structural components, quercetin (Q), silybin (SB), gallic acid, and gallic acid methyl ester. Their multi-step pH-dependent anodic behaviour, first oxidation followed by oxidation of the hydroxyl groups at ring A, is similar to Q and SB. The galloyl substitution significantly improved the antioxidant potential of SB compared to Q, and brought useful knowledge about the antioxidant activity of Q and SB monogalloyl esters.

Keywords: Galloylquercetin, Galloylsilybin, Flavonol, Flavonolignan, Glassy carbon electrode, Oxidation

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1 Introduction
Polyphenols belong to the most abundant group of antioxidants in the human diet. Study of their antioxidant properties began around 1995, which was also the starting point for a number of epidemiological studies showing a relationship between polyphenol-rich food consumption and cancer or cardiovascular diseases incidence [1]. The antioxidant effects of polyphenols are due to the scavenging of a wide range of reactive oxygen [2,3], nitrogen [2,4,5], and chlorine [6] species; metal chelating properties [7,8] as well as the modulation of activity of enzymes involved in the oxidative stress [9,10]. However, the prooxidant effects of polyphenols have also been described in vitro under certain experimental conditions [11–13]. Recent data showed that in general the equilibrium of antioxidant and prooxidant effects plays an important role in the biological activity of polyphenols [14].

Several methods for the study of redox properties and antioxidant capacity of polyphenols have been reported [15–19]. Electrochemical techniques, namely cyclic, differential pulse, square-wave voltammetry and amperometry are commonly used [17,18,20–23]. Understanding of the oxidation mechanisms also provides insight into the behaviour (e.g. biotransformation) of polyphenolic compounds in the human organism and it is of fundamental importance for evaluation of their antioxidant and prooxidant activities.

Flavonoids are polyphenols composed of two benzenoid rings, ring A and ring B, and one pyran ring C fused to ring A, and linked to ring B by a C–C bridge (Scheme 1). Although many contradictions exist regarding the oxidation behaviour and the structure-activity relationship of flavonoids, in general, the electrooxidation mechanism proceeds in two or more steps. First, at low positive potentials, a two electron-two proton oxidation of the catechol moiety, at \( E_{p} \approx +0.2 \) V, followed by the oxidation of the resorcinol moiety at a more positive potential, at \( E_{p} \approx +0.8 \) V, vs. Ag/AgCl [24–28]. In addition to the described two oxidi-
dation steps, an oxidation product formed at the potential of the catechol moiety oxidation, at $E_p \approx +0.2 \, \text{V}$, can be oxidised at $E_p \approx +0.5 \, \text{V}$ [29].

The Q derivatives, rutin [28,30], isoquercitrin [31], and taxifolin [28], have been also studied electrochemically. The SB electrochemical behaviour occurred in two consecutive oxidation steps [13,32–34], the oxidation of the 20-hydroxyl group, at $E_p \approx +0.5 \, \text{V}$ (Scheme 1), followed by the oxidation of the 5-OH group at ring A, at a higher potential $E_p \approx +0.85 \, \text{V}$ [13].

Recently, besides natural flavonoids also semisynthetic flavonoids have been investigated, e.g. green tea polyphenols and their analogues and prodrugs [35]. The conjugates prepared and several structural modifications of flavonoids lead to new biological activities and pharmaceutical potential improvement. Two recently synthesized Q and SB galloyl esters, quercetin-3-O-gallate (QG) [36], and silybin-3-O-gallate (SBG) [37] are of particular interest due to possible antioxidant activity improvement and water solubility, owing to the galloyl moiety substitution. In this paper the electrochemical behaviour of Q and SB galloyl esters is described for the first time.

The monogalloyl substitution effect on Q and SB, the redox behaviour of QG and SBG, and of their structural components, gallic acid (GA) and gallic acid methyl ester (MeGA), on a glassy carbon electrode, using voltammetric techniques under a wide range of pH, was investigated, enabled the proposal of an oxidation mechanism for QG and SBG, and brought useful knowledge about the antioxidant activity of Q and SB monogalloyl esters.

## 2 Experimental

### 2.1 Chemicals

Silybin (SB) was kindly provided by Dr. L. Cvak (TAPI Galena, IVAX Pharmaceuticals, Opava, CZ) and silybin-3-O-gallate (SBG) was prepared as described previously [37]. Quercetin (Q), gallic acid (GA) and gallic acid methyl ester (MeGA) were from Sigma-Aldrich (St. Louis, MO, USA), and quercetin-3-O-gallate (QG) was prepared according to the previously published protocol [36]. Buffer chemicals were from Sigma-Aldrich.

Analytical-grade reagents and purified water from a Millipore Milli-Q system (conductivity <0.1 \mu\text{s/cm}) were used for the preparation of 0.1 M supporting electrolytes: HCl/KCl (pH 1.2, 2.02), HAcO/NaAcO (pH 3.4, 4.3, 5.4), NaH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 6.08, 7, 8.05), Na$_2$B$_4$O$_7$·10 H$_2$O/NaOH (pH 9.25), NaOH/KCl (pH 11, 12.04).

Stock solutions of 1 mg/mL were prepared in methanol and stored at 4°C. The working solutions were prepared by diluting the stock solution with the supporting electrolyte.

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Scheme 1. Chemical structures: (QG) quercetin-3-O-gallate, (SBG) silybin-3-O-gallate, (Q) quercetin, (SB) silybin, (GA) gallic acid and (MeGA) gallic acid methyl ester.
Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pipettes (Rainin Instrument Co. Inc., Woburn, USA). The pH measurements were carried out with a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1°C).

2.2 Electrochemical Measurements

All electrochemical measurements were performed using a Ivium potentiostat in combination with IviumSoft program version 1.9 (Ivium Technologies, Eindhoven, The Netherlands) in a three-electrode setup, glassy carbon electrode (GCE, \(d=1.0\) mm) (Bio-Logic SAS, France) as working electrode, Ag/AgCl (3 M KCl) electrode as reference and platinum counter electrode in a 0.5 mL one-compartment electrochemical cell (Bio-Logic SAS, France). The experimental conditions were: for cyclic voltammetry (CV) scan rate 50 and 1000 mVs\(^{-1}\), for differential pulse (DP) voltammetry pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mVs\(^{-1}\), and for square wave (SW) voltammetry frequency \(f=50\) Hz, \(\Delta E_s=2\) mV, pulse amplitude 50 mV, \(v_{ef}=100\) mVs\(^{-1}\).

Before each electrochemical experiment the GCE was always polished using diamond spray (particle size 3 \(\mu\)m) (Kernet Int., UK). After polishing, the electrode was rinsed thoroughly with Milli-Q water and placed into the supporting electrolyte and voltammograms were recorded until steady state baseline voltammograms were obtained.

2.3 Acquisition and Presentation of Voltammetric Data

SW and DP voltammograms presented were baseline-corrected using the moving average application with a step window of 5 mV included in IviumSoft program version 1.9. This mathematical treatment improves the visualization 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

The anodic behaviour of QG and SBG was investigated using CV, DP and SW voltammetry at GCE over a wide pH range. In order to identify the redox active centres and propose an oxidation mechanism for QG and SBG, the electrochemical oxidation of GA, MeGA, Q, SB, Scheme 1, in 0.1 M phosphate buffer (pH 7.0) by DP voltammetry, was first studied.

3.1 Oxidation of GA, MeGA, Q, SB

The electrochemical oxidation of 5 \(\mu\)M GA, MeGA, and Q, and 10 \(\mu\)M SB, Scheme 1, was investigated performing successive DP voltammograms from 0 to +1.2 V, Figure 1 and Table 1. The second DP voltammograms recorded in the same solutions and without cleaning the GCE, showed a decrease of oxidation peak currents, due to adsorption on the electrode surface of the polymeric oxidation products formed, which can lead to the electrode passivation.

DP voltammograms of GA and MeGA showed one oxidation peak 1a, at \(E_{p1a}=+0.12\) V (Figure 1A,B). The oxidation mechanism and antioxidant capacity of GA and selected alkylgallates in neutral aqueous medium, showed that GA is first oxidized to a semiquinone radical cation.
by an electron transfer process. Then the radical cation loses a proton to form the semiquinone radical, followed by a second electron transfer to the quinone cation, which is deprotonated to quinone [40], the redox process of peak 1a involving two electrons-two protons transfer.

However, for GA, another oxidation peak 2a, at $E_{p2a} = +0.49$ V, was observed but the peak 2a current (Figure 1A), was significantly lower than the peak 1a current. The peak 2a is also found in alkylgallates, but only under specific experimental conditions [38–40].

DP voltammograms of Q showed the first oxidation peak 1a, at $E_{p1a} = +0.16$ V, and the second oxidation peak 2a, at $E_{p2a} = +0.74$ V (Figure 1C). The first oxidation peak 1a is related to the oxidation of the catechol moiety, ring B, in a two electron-two protons transfer, and the second oxidation peak 2a is attributed to the oxidation of the resorcinol moiety, ring A [24,29]. An oxidation product was also formed at the potential of the catechol group oxidation, which has been identified using spectroelectrochemical methods and exhaustive electrolysis under anaerobic conditions [29].

DP voltammograms of SB showed the first oxidation peak 1a, at $E_{p1a} = +0.47$ V, and the second oxidation peak 2a, at $E_{p2a} = +0.74$ V (Figure 1D). The first oxidation peak 1a is attributed to the oxidation of the 20-hydroxyl group at ring E, and the second oxidation peak 2a, at a more positive potential, corresponds to the oxidation of the 5-hydroxyl group of the resorcinol group, ring A. These results are in agreement with experimental and computational data [13] and the oxidation mechanism proposed for SB in complex system of silymarin [32], and partially also with pulse radiolysis measurement [41].

The second DP voltammograms of SB showed two new oxidation peaks, peak 3a, at $E_{p3a} = +0.12$ V, and peak 4a, at $E_{p4a} = +0.19$ V, corresponding to the oxidation of the electroactive SB oxidation products, probably formed in the oxidation of the phenol-like, ring E, and the resorcinol, ring A, moieties.

### Table 1. Oxidation peak potentials obtained by DP voltammetry in 0.1 M phosphate buffer (pH 7.0).

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>Peak 1a</th>
<th>Peak 2a</th>
<th>Peak 3a</th>
<th>Peak 4a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid (GA)</td>
<td>0.10 V</td>
<td>0.49 V</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gallic acid methyl ester (MeGA)</td>
<td>0.13 V</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Quercetin (Q)</td>
<td>0.16 V</td>
<td>0.74 V</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Silybin (SB)</td>
<td>0.47 V</td>
<td>0.74 V</td>
<td>0.12 V</td>
<td>[a] 0.19 V</td>
</tr>
<tr>
<td>Quercetin-3-O-gallate (QG)</td>
<td>0.15 V</td>
<td>0.27 V</td>
<td>0.82 V</td>
<td>–</td>
</tr>
<tr>
<td>Silybin-3-O-gallate (SBG)</td>
<td>0.16 V</td>
<td>0.49 V</td>
<td>0.78 V</td>
<td>–</td>
</tr>
</tbody>
</table>

[a] Oxidation products, second scan

3.2 Oxidation of Quercetin-3-O-Gallate and Silybin-3-O-Gallate

The electrochemical behaviour of 50 μM QG and 20 μM SBG was first investigated by CV and SW voltammetry in phosphate buffer (pH 7.0) (Figures 2, 3, and 4). In SW voltammetry the current is sampled in both positive and negative pulses, and the peaks corresponding to the oxidation and reduction of the electroactive species at the electrode surface can be obtained in the same experiment [42], enabling the confirmation of the results obtained by CV.

CVs of QG showed three oxidation peaks, $E_{p1a} = +0.19$ V, $E_{p2a} = +0.27$ V and $E_{p3a} = +0.87$ V (Figure 2A). The reversibility of the QG anodic reactions at peaks 1a and 2a was only detected using, in the potential range $-0.2$ to $+0.4$ V, a high scan rate of 1.0 V s$^{-1}$ (Figure 3A). If the electrode was polarized at more than $+1.1$ V, it was impossible to observe the reversible peaks, because a poly-

![Fig. 2. CVs in 0.1 M phosphate buffer (pH 7.0): (A) 50 μM QG at $v = 25$ mV s$^{-1}$ and (B) 20 μM SBG at $v = 50$ mV s$^{-1}$; (—) first, (····) second and (---) third scans, (--) supporting electrolyte.](image-url)
meric film is formed on electrode surface, already observed for polyphenols, including flavonols and flavonolignans [13,43,44], and no electroactivity of QG oxidation products was detected. SW voltammetry confirmed the three oxidation steps of QG, peak 1a, at $E_{p1a} = +0.19$ V, peak 2a, $E_{p2a} = +0.25$ V, and peak 3a, $E_{p3a} = +0.86$ V (Figure 4A). The reversibility of peaks 1a/1c and 2a/2c of QG was confirmed by plotting the forward and backward components of the total current (Figure 4A).

Similarly to QG the CVs of SBG also showed three oxidation peaks, $E_{p1a} = +0.17$ V, $E_{p2a} = +0.55$ V and $E_{p3a} = +0.83$ V (Figure 2B), and in subsequent scans the currents decreased due to adsorption processes and/or the formation of a polymeric film on the electrode surface, and no electroactive SBG oxidation products were formed. The reversibility of the SBG peak 1a anodic reaction was also only detected using, in the potential range $0.2$ to $+0.4$ V, a high scan rate of $1.0$ V s$^{-1}$ (Figure 3B). SW voltammetry also confirmed the three oxidation steps of SBG, peak 1a, at $E_{p1a} = +0.20$ V, peak 2a, $E_{p2a} = +0.55$ V, and peak 3a, $E_{p3a} = +0.84$ V (Figure 4B), and the reversibility of peak 1a/1c of SBG was also confirmed by plotting the forward and backward components of the total current (Figure 4B). The peaks 2a and 3a of SBG are irreversible under the selected experimental conditions in agreement with CV.

DP voltammetry in 5 μM QG was performed over a wide supporting electrolyte pH range between 1.2 and 12.0. At acidic conditions, pH 3.4, the QG oxidation occurs in three consecutive steps, at potentials $E_{p1a} = +0.39$ V, $E_{p2a} = +0.47$ V and $E_{p3a} = +1.0$ V. In 3.4 < pH < 10 the peaks 1a and 2a are shifted to less positive potentials with increasing pH, and the highest peak currents were at the lowest pH (Figure 5A). The slope of ~60 mV per pH unit of $E_{p1a}$ and $E_{p2a}$ vs. pH, and $W \approx 60$ mV, shows that the peaks 1a and 2a oxidation involved two electrons-two protons transfer (Figure 5B), which is in agreement with oxidation mechanism of catechol [45] and galloyl [38] moieties. For pH > 10, the peak 1a and 2a oxidation potentials become pH-independent explained considering that QG undergoes chemical deprotonation in alkaline media (Figure 5B). At more
positive potentials, for 1.2 < pH < 8.0, a new oxidation peak 3a occurred, following a linear dependence with pH, $E_{p3a} = -0.06 \text{ pH} + 1.24 \text{ V}$, and $W_{1/2} \approx 90 \text{ mV}$, indicating that the oxidation occurs with one electron-one proton transfer.

The QG first oxidation occurs at the catechol and the galloyl moieties followed by the oxidation at the resorcinol moiety. The QG three-step anodic reactions (Figure 1E), are accompanied by a proton loss and formation of a semi-quinone radical. The catechol group in epicatechin gallate is oxidized first [46], whereas the galloyl moiety oxidation occurs at a slightly more positive potential and the QG oxidation probably undergoes a similar process.

DP voltammograms were also obtained for 10 μM SBG in supporting electrolytes with different pH values. In 0.1 M acetate buffer (pH 3.4) were observed three oxidation steps, peak 1a, at $E_{p1a} = +0.38 \text{ V}$, peak 2a, at $E_{p2a} = +0.65 \text{ V}$ and peak 3a, at $E_{p3a} = +1.03 \text{ V}$ (Figure 6A). Increasing the pH, the peaks oxidation potential was shifted linearly to less positive potentials until pH 8. For pH > 8, the oxidation reaction becomes pH-independent due to deprotonation (Figure 6B). The highest peak currents were also in the lowest pH. The slope of ~60 mV per pH unit of $E_{p1a}$, $E_{p2a}$ and $E_{p3a}$ vs. pH, showed that the oxidation involved the same number of electrons and protons (Figure 6B). The first oxidation step occurs with two electrons-two protons transfer corresponding to GA oxidation [42]. The second and third oxidation peaks with $W_{1/2} \approx 100 \text{ mV}$, indicated involved one electron-one proton transfer, in agreement with the oxidation mechanism of SB [13] and silymarin [32].

Analogously to QG, the SBG oxidation also occurs in a cascade mechanism, related first to the galloyl moiety oxidation involving two electrons, followed by the two steps oxidation on the 2-hydroxyl group and the resorcinol group of the SB moiety, involving one electron-one proton transfer each (Figure 1F).

The quinones formation via semi-quinone radical intermediates and also more complex structures formation, like polymeric films, onto GCE at higher potentials, occur in QG and SBG oxidation.

The antioxidant capacity of polyphenols can be deduced from the potential of the first oxidation in the first anodic scan, where the antioxidant capacity of the oxidized compounds is negatively associated with their oxidation potential. Thus, the galloyl substitution significantly improved the antioxidant potential of SB, which is naturally a ‘weak’ antioxidant. In the case of QG, the galloyl moiety oxidation occurs at a potential close to the catechol group oxidation potential, indicating no qualitative improvement in the Q antioxidant activity, which is generally considered a ‘strong’ antioxidant, after galloyl substitution.

4 Conclusions

The QG and SBG anodic reactions occurred in a multi-step pH-dependent mechanism, and were compared with the electrochemical behaviour of their structural components, Q, SB, GA and MeGA. The first oxidation step for QG and SBG is associated with the galloyl and/or catechol moieties oxidation, and is followed by the hydroxyl groups oxidation at ring A, with similar oxidation reactions as Q and SB. The results brought useful knowledge about oxidation and antioxidant activity of Q and SB monogalloyl esters, will be relevant in studies focusing on modulation of biological activity of flavonoids and other bioactive compounds using galloyl substitution, and in electroanalytical applications.

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Electrochemical Oxidation of Quercetin and Silybin Galloyl Esters at GCE


