Electrochemical Oxidation Mechanisms of the Antioxidants Daidzein and 7-Hydroxy-4-chromone

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Abstract
The electrochemical behavior of antioxidants daidzein (DZ) and 7-hydroxy-4-chromone (7-OH-4-CHM) was investigated. The oxidation of DZ is irreversible, pH-dependent, and occurs in two steps, the first of 4′-OH on the B-ring and the second on the 7-OH on the A-ring. The oxidation of 7-OH-4-CHM occurs on the 7-OH on the A-ring, is irreversible, pH-dependent and proceeds in a single step. The DZ diffusion coefficient was calculated in pH 7.0 phosphate buffer to be $D_{DZ} = 8.1 \times 10^{-5}$ cm$^2$ s$^{-1}$. The detection limits of DZ and 7-OH-4-CHM was calculated as $LOD_{DZ} = 0.08 \mu M$ and $LOD_{7-OH-4-CHM} = 0.13 \mu M$.

Keywords: Flavonoids, Daidzein, Oxidation, Antioxidant activity, Glassy carbon electrode

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1 Introduction

Recently increasing interest has been observed in naturally occurring pharmatherapeutic compounds. These compounds have important health protecting properties, such as antioxidant, anti-inflammatory, antiallergic, antibacterial, antiparasitic antifungal, antimicrobial, antidiabetic and anticarcinogenic properties. It is well known that antioxidants reduce the risk of chronic diseases, including coronary diseases and cancer, and anticarcinogenic substances, among various effects, can induce apoptosis destroying tumor cells. [1,2].

Flavonoids are one of the more important groups of natural pharmatherapeutic compounds and the largest family of phenolic compounds [1], comprising several sub-classes, which include a great number of compounds with different chemical properties: flavonols, flavones, flavanols and isoflavones. Flavonoids consist of two benzene rings linked by an oxygen containing heterocycle, Scheme 1, and are characterized by the number of hydroxyl groups on the A- and B-rings. The antioxidant activity of flavonoids resides in their aromatic OH groups, which may dominate, and their metal chelating properties [3]. Isoflavones are flavonoids occurring naturally in soybean and are also found in plants that humans generally do not eat, such as red clover and kudzu [4,5].

Daidzein (DZ) (7,4′-dihydroxyisoflavone) is an isoflavone found in soybeans, with a wide spectrum of physiological and pharmacological functions (Scheme 1) including antioxidant, anti-inflammatory, cell cycle arrest and estrogen-like biological activities in humans [6–10]. The antioxidant effect of isoflavones has been demonstrated in many biologically relevant systems and studies of the structure activity relationship have shown that the 4′-OH on the B-ring is essential in scavenging free radicals, whereas the effect of the 7-OH on the A-ring is less significant [10].

As result of the importance of DZ in biological systems, different methods have been applied for the determination of DZ in foods and biological fluids including gas chromatography (GC) [11], GC-MS [12] high-performance liquid chromatography (HPLC) [13–15], liquid chromatography (LC) [16], capillary zone electrophoresis (CZE) with UV detection [17], electrochemical detection [18] capillary electrophoresis with electrochemical detection [19], and hydrodynamic and cyclic voltammetry [4,5,18–21]. However, more recently, there has been a tendency for the use of less costly techniques and a reduction in the use of organic solvents since they result in high ecological costs.

Investigations of the redox behavior of flavonoids using electrochemical techniques have the potential for providing valuable insights into the redox reaction mechanisms of these molecules that are very relevant for evaluating their antioxidant activity. Voltammetric methods, due to their high sensitivity, have been used to study the redox behavior of various flavonoids [3,22–25]. In the present paper, the electrochemical behavior of DZ, for a wide pH range between 2 and 12, using cyclic, square wave and differential pulse voltammetry at a glassy carbon electrode was investigated for the first time and, compared...
with the electrochemical behavior of 7-hydroxy-4-chromone (7-OH-4-CHM), a compound structurally similar to DZ but without the B-ring and the 4'-OH group (Scheme 1).

The oxidation mechanisms of DZ and 7-OH-4-CHM were investigated at different pHs, and the information obtained may play a crucial role in understanding the antioxidant properties of these compounds as well as their metabolism in biological systems. A new electroanalytical procedure for the determination of DZ using square wave voltammetry and of 7-OH-4-CHM using differential pulse voltammetry was developed.

2 Experimental

2.1 Materials and Reagents

Daidzein (DZ) and 7-hydroxy-4-chromone (7-OH-4-CHM) were obtained from Sigma and used without further purification. Stock solutions of 1.00 mM of DZ, in ethanol, and 7-OH-4-CHM, in deionized water/ethanol (2:1, v/v), were prepared and stored at 4°C. All supporting electrolyte solutions (Table 1) were prepared using analytical grade reagents and purified water from a Milli-Q system (conductivity ≤0.1 µS/cm⁻¹). Micro-volumes 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 micro-pH 2001 pH-meter with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1°C).

2.2 UV-vis Absorption

Absorption spectra were recorded using a UV-vis spectrophotometer SPECORD S100 from Carl Zeiss Technology with Win-Aspect software and a 10 mm quartz cuvette. The experimental conditions for absorption spectra were: integration time 25 ms and accumulation 1000 points. All UV-vis spectra were measured from 200 nm to 400 nm. Plots of adsorption at different wavelengths versus apparent pH values of sample the solution generated sigmoid curves. The pKₐ constants were determined for DZ and 7-OH-4-CHM on the basis of the absorbance at 336 nm using the Henderson–Hasselbach equations [26].

2.3 Voltammetric Parameters and Electrochemical Cells

Voltammetric experiments were carried out using a μAutolab running with GPES 4.9 software, Metrohm/Autolab, Utrecht, The Netherlands. Measurements were carried out using a glassy carbon working electrode (GCE) (d = 1.5 mm), a Pt wire counter electrode, and an Ag/AgCl (3 M KCl) as reference electrode, in a 1 mL one-compartment electrochemical cell. The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms, and scan rate 5 mVs⁻¹. For square-wave (SW) voltammetry the experimental conditions were frequency 25 Hz and potential increment 2 mV, corresponding to an effective scan rate of 100 mVs⁻¹.

The GCE was polished using diamond spray (particle size 1 µm, Kement, Kent, UK) before every electrochemical assay. After polishing, the electrode was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer supporting electrolyte and various DP voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

Table 1. Supporting electrolytes, 0.1 M ionic strength.

<table>
<thead>
<tr>
<th>pH</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
<td>HCl + KCl</td>
</tr>
<tr>
<td>3.4</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>4.4</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>5.3</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>6.1</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
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</tr>
<tr>
<td>7.9</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>9.1</td>
<td>NaOH + Na₂B₂O₄</td>
</tr>
<tr>
<td>11.6</td>
<td>NaOH + KCl</td>
</tr>
</tbody>
</table>

Scheme 1. Chemical structures: (a) Flavonoids, (b) DZ and (c) 7-OH-4-CHM.
2.4 Acquisition and Presentation of Voltammetric Data

All DPVs presented were background-subtracted and baseline-corrected using the moving average application with a step window of 2 mV included in the GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artifact, although the peak current 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 graphs were determined from the original untreated voltammograms after subtraction of the base line.

3 Results and Discussion

3.1 Cyclic Voltammetry

The oxidation behavior of DZ and 7-OH-4-CHM at a GCE was investigated in buffer electrolytes with different pHs, by cyclic voltammetry (CV) at 100 mVs⁻¹, and it was found that all functional OH groups attached to the ring structures were electroactive.

The DZ molecule possesses two phenol moieties and is planar (Scheme 1).

CVs of 300 μM DZ in acid media pH 4.5 0.1 M acetate buffer showed on the first scan two consecutive well-separated irreversible oxidation peaks. Both peak 1, at \( E_{pa1} = +0.78 \) V, and peak 2, at \( E_{pa2} = +1.0 \) V, correspond to the oxidation of the OH groups in the molecule (Figure 1A). Successive scans without polishing the electrode between the cycles clearly demonstrated that the electrode surface is rapidly blocked. This occurs due to the polymerization of DZ oxidation product (Figure 1A).

CVs of DZ in pH 7.0 0.1 M phosphate buffer showed the anodic peak 1, at \( E_{pa1} = +0.56 \) V and the peak 2, at \( E_{pa2} = +0.86 \) V, and the peak currents decreased in successive scans (Figure 1B). In comparison with the results in acid media (Figure 1A), the oxidation potentials of the peaks 1, and 2, were shifted to less negative values with increasing pH. Since adsorption of DZ and/or its oxidation product is very strong in acid electrolytes, further studies were carried out in pH 7.0 0.1 M phosphate buffer. CVs were obtained at different scan rates, from 10 to 800 mVs⁻¹ in a 300 μM DZ solution, and peak 1, and 2, currents were linearly dependent on square root of the scan rate, consistent with diffusion-limited oxidation of a solution species.

The oxidation mechanism of DZ for peak 1 (Scheme 2) is based on the data and slope of Tafel plot (log I vs. E). Considering, scan rate \( v = 500 \text{ mVs}^{-1} \), the slope of the Tafel plot, slope = \( \alpha_s/R/2.3RT \), gives \( \alpha_s = 0.65 \). The number of electrons participating in the electrode reaction can be calculated since \( |E_{pa1} - E_{p2a}| \approx 87 \) mV for peak 1, and for an irreversible system \( |E_{pa1} - E_{p2a}| = 47.7/(\alpha_sn) \) [27], so \( n = 1 \) for peak 1.

The peak current in amperes for a diffusion-controlled irreversible system is given by the equation:

\[
I = (nFADC/RT)^{1/2}v^{1/2}
\]
where \( n \) is the number of electrons transferred during the first oxidation of DZ, \( A \) is the electrode area in cm\(^2\), \( D_0 \) is the diffusion coefficient in cm\(^2\)s\(^{-1}\), [O] is the concentration in mol/cm\(^3\) and \( v \) is in Vs\(^{-1}\). The value of \( D_{DZ} \) was obtained plotting \( I_{pa} \) vs. \( v^{1/2} \). For the measured slope of \( 5.6 \times 10^{-6} \)A/(Vs\(^{-1}\))\(^{1/2} \) the diffusion coefficient of DZ in pH 7 0.1 M phosphate buffer is \( D_{DZ} = 8.1 \times 10^{-5} \) cm\(^2\)s\(^{-1}\). The GCE electroactive area was determined from a plot of \( I_{pa} \) vs. \( v^{1/2} \) using a solution of 0.5 mM hexacyanoferrate and the diffusion coefficient of hexacyanoferrate in phosphate buffer of \( D_0 = 7.35 \times 10^{-6} \) cm\(^2\)s\(^{-1}\). The electroactive area of GCE was determined to be 0.011 cm\(^2\).

To clarify the physicochemical properties of the DZ and its oxidation mechanism in aqueous solution, the investigation of the redox behavior of 7-OH-4-CHM with one phenol moiety on the A-ring but without the B-ring and the 4’-OH group, due to a similar structure was undertaken.

CVs of 50 \( \mu \)M 7-OH-4-CHM in pH 7.0 0.1 M phosphate buffer, at a scan rate \( v = 100 \) mVs\(^{-1}\) (Figure 1C) show just one irreversible anodic peak 1a at \( E_{pa} = +0.83 \) V, scanning in the negative direction, no reduction peak was observed, and a decrease of the oxidation current with the number of successive scans was obtained.

The oxidation mechanism of 7-OH-4-CHM proposed (Scheme 3) is based on the data and slope of the Tafel plot (log \( I \) vs. \( E \)). From the slope of the Tafel plot \( \alpha = 0.67 \) for peak 1a, at \( v = 500 \) mVs\(^{-1}\). And since \( |E_{pa} - E_{pa/2a}| \approx 50 \) mV, \( n = 1 \) was calculated.

Considering DZ and 7-OH-4-CHM chemical structure the differences in the CVs of the two compounds are explained. The hydroxyl group on the DZ molecule on B-ring, Scheme 1, is absent on the 7-OH-4-CHM molecule. The peak 1a of the DZ, corresponds to the oxidation of the 4’-OH of B-ring, that occurs at a less positive potential, so 4’-OH is more easily oxidized and consequently has a better reducing power than the 7-OH, in agreement with previously reported results [10], explaining DZ antioxidant activity in vitro. DZ peak 2a, corresponds to the
oxidation of the 7-OH on A-ring, that occurs at a high positive potential, the same potential of peak 1a of 7-OH on A-ring of 7-OH-4-CHM.

3.2 Differential Pulse Voltammetry. Effect of pH

The DZ antioxidant action mechanism is due to the capacity of the phenolic compound to scavenge radicals by electron transfer, and the oxidation of phenolic compounds is pH-dependent.

The effect of pH on the electrochemical oxidation of 2.5 μM DZ (Figure 2A) and 50 μM 7-OH-4-CHM (Figure 4A) was studied by DP voltammetry in supporting electrolytes with 2 < pH < 12 and 0.1 M ionic strength (Table 1).

The DP voltammograms of DZ showed that the oxidation occurs in two steps, peaks 1a and 2a (Figure 2A). For 2.0 < pH < 8.0 the peak 1a potential shifted to more negative values with increasing pH (Figure 2A). In the $E_{pa}$ vs. pH plot (Figure 2B), the slope of the dotted line, $-59$ mV per pH unit, showed that the mechanism of peak 1a involves the same number of electrons and protons. The number of electrons transferred was determined by the peak width at half height $W_{1/2} \approx 90$ mV, corresponding to an electrochemical reaction involving the transfer of one electron. So, DZ peak 1a corresponds to the oxidation of the 4'-OH group at the B-ring and occurs with the transfer of one electron and one proton. For pH > 8, the oxidation peak 1a is pH independent indicating a mechanism involving only one electron (Figure 2A).

Peak 2a corresponds to DZ oxidation on the 7-OH group of the A-ring. The potential shifts to more negative values with increasing pH, and only occurs for electrolytes with pH < 8.0 (Figure 2A and 2B). In the $E_{pa}$ vs. pH plot (Figure 2B), the slope of the dotted line, $-59$ mV per pH unit, shows that the mechanism involves the same number of electrons and protons. Taking into consideration that the width at half height of the DZ oxidation peak 2a was $W_{1/2} \approx 70$ mV, it is concluded that the oxidation process involves the transfer of one electron and one proton.

The plot of peaks 1a and 2a current versus pH (Figure 2B), shows that the DZ peak currents are much higher for 2.0 < pH < 5.0, due to the effect of pH on ionization of OH groups. In acid media the OH groups increase DZ hydrophobicity and adsorption on the hydrophobic GCE surface. At neutral and alkaline pH, the OH groups are almost or fully ionized (deprotonated) increasing DZ hydrophilicity and consequently decreasing DZ adsorption on the GCE surface.

The strong adsorption of DZ on the GCE surface was investigated by two procedures. In the first, after many scans in the DZ solution in pH 4.5, the electrode was washed with a jet of deionized water and transferred to the supporting electrolyte, where DP voltammograms in buffer solution showed peaks 1a and 2a. In the second, the electrode was immersed in a 50 μM DZ solution at different pHs 4.5, 7.0 and 9.0 for 10 min, and after the electrode was washed with a jet of deionized water and trans-
ferred to the buffer solution peak 1a, with higher current in acid media, appeared in all DP voltammograms. The DP voltammograms of 50 μM 7-OH-4-CHM in different electrolytes showed only one irreversible pH-dependent oxidation peak 1a, the potential shifted to more negative values with increasing pH (Figure 3A), and the highest peak current was at pH 2.0 (Figure 3B). The adsorption of 7-OH-4-CHM and/or its oxidation products on the electrode surface was very strong in acid media.

For 2.0 < pH < 8.0 the pH dependence was linear with slope \(-59 \text{ mV per pH unit}\) (Figure 3A) so the same number of electrons and protons is involved in the 7-OH-4-CHM oxidation (Figure 3B) and as the width at half-height of peak 1a is \(W_{1/2} \approx 100 \text{ mV}\), occurs with transfer of one electron and one proton.

For pH > 8, as the 7-OH-4-CHM undergoes chemical deprotonation in alkaline electrolytes, and the oxidation peak is pH-independent with a mechanism involving only one electron.

3.3 Oxidation Mechanism of DZ and 7-OH-4-CHM

The DZ molecule contains two groups 4'-OH, at B-ring, and 7-OH, at A-ring, while the 7-OH-4-CHM molecule only contains one group 7-OH, at A-ring.

The peak 1a of DZ corresponds to the oxidation of 4'-OH group, whereas DZ peak 2a to the oxidation of the 7-OH group, in agreement with the 4'-OH group being more easily oxidized than the 7-OH group [10].
The oxidation of phenols (OH groups) involves the formation of a phenoxy radical, that can initiate polymerization, leading to adsorbed products on the electrode surface [28–31], and the radical can also be oxidized to a quinone that is reversibly reduced, in another pathway [32,33].

Based on the results described oxidation mechanisms for DZ (Scheme 2) and 7-OH-4-CHM, Scheme 3, were proposed. In DZ and 7-OH-4-CHM oxidations, the phenoxy radical coupling forming polymeric products corresponds to the main reaction since no cathodic peak was observed by CV.

3.4 Analytical Determination

For the electroanalytical determination of DZ and 7-OH-4-CHM the oxidation peak 1a was chosen. Between measurements the GCE surface was always polished in order to ensure a clean electrode surface and to avoid the adsorption of DZ and 7-OH-4-CHM oxidation products.

Calibration curves were plotted for peak 1a current vs. bulk concentrations of 0.0–1.0 μM of DZ in pH 4.0 and of 7-OH-4-CHM in pH 4.5, 0.1 M acetate buffer (Figure 4). For each concentration three measurements were performed. Good linearity was found between peak current and concentration described by the equations:

\[ I_{pa} = 0.12 \text{ [DZ mM]}^{-1.39 \times 10^{-10}} \]

Fig. 5. Absorption spectra at different pHs: (A) DZ and (B) 7-OH-4-CHM, arrows indicate pH increase. Inset: Absorption at 336 nm vs. pH.
Figure 5A) was calculated.

In acid media the limits of detection, pH and concentration, were investigated and optimized.

7-OH-4-CHM, in agreement with its better antioxidant dependent, occurs in a single step corresponding to the oxidation of DZ in the range 225–400 nm, while in neutral solution the spectrum exhibited two adsorption bands with maxima at 250 and 336 nm (Figure 5A). However, in alkaline solution the DZ spectrum showed a new absorption band with maximum at 336 nm (Fig-ure 5A). The same UV-vis behavior was observed for 7-OH-4-CHM and the pKa was 7.5 determined from Figure 5B. The results obtained for DZ and 7-OH-4-CHM are in agreement with the 7-OH group on the A-ring being more acidic than the 4'-OH on the B-ring [5,6].

The parameters to define the sensitivity were calculated and the value obtained for the limit of detection, LODDZ = 0.08 μM and LOD7-OH-CHM = 0.13 μM, was based on three times the noise level.

3.5 Determination of the pKa Values by UV-vis Spectrophotometry

The pKa values of DZ and 7-OH-4-CHM, Figure 5, were determined spectrophotometrically in 0.1 M ionic strength aqueous solution at different pHs (Table 1).

In acid media, the DZ spectrum in the range 225–400 nm, while in neutral solution the spectrum exhibited a new absorption band with maximum at 336 nm (Figure 5A). However, in alkaline solution the DZ spectrum presented again two adsorption bands with maxima at 250 and 336 nm. The pKa1 = 7.5 of DZ at 336 nm (Inset Figure 5A) was calculated.

The same UV-vis behavior was observed for 7-OH-4-CHM and the pKa = 7.5 determined from Figure 5B. The results obtained for DZ and 7-OH-4-CHM are in agreement with the 7-OH group on the A-ring being more acidic than the 4'-OH on the B-ring [5,6].

4 Conclusions

The electrochemical study showed that DZ and 7-OH-4-CHM undergo oxidation at a GCE and the electron transfer mechanisms are proposed. The oxidation of DZ is complex, pH-dependent, and all steps are irreversible, strongly absorbs on the electrode surface in acid media and the two oxidation peaks correspond to the oxidation of the two phenol groups. The diffusion coefficient of DZ was calculated in pH 7.0 phosphate buffer to be D = 8.1 × 10⁻⁵ cm² s⁻¹. The oxidation of 7-OH-4-CHM is also pH-dependent, occurs in a step corresponding to the oxidation of the 7-OH group. The DZ is more easily oxidized, and consequently has a better reducing power than 7-OH-4-CHM, in agreement with its better antioxidant activity.

The electroanalytical experimental conditions, buffer pH and concentration, were investigated and optimized. In acid media the limits of detection, LODDZ = 0.08 μM and LOD7-OH-CHM = 0.13 μM, were obtained.

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