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Electrochemistry of catechol-containing flavonoids

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Abstract: The electrochemical properties of four structurally related flavonoids, quercetin, quercetin-3-O-rhamnose (quercitrin), quercetin-3-O-rutinose (rutin) and luteolin were investigated. These flavonoids were shown to undergo homogenous chemical reactions following oxidation at a glassy carbon electrode. These reactions were studied using cyclic voltammetry and rotating ring-disk voltammetry. Both first-order and zero-order processes were observed. The rate of the zero-order process was strongly dependent on the substituent at the C-3 position of the flavonoid. The rate of the first-order process was independent of substitution. Two products were observed using liquid chromatography. These products did not correspond to previously reported products of enzymatic oxidation. The products were not stable under conditions for isolation.

Keywords: *Flavonoids; oxidation mechanism; electrochemistry of quercetin; EC reaction kinetics.*

Introduction

Flavonoids are benzo-γ-pyrone derivatives widely distributed in nature. The literature on the electrochemistry of flavonoids is limited [1–5]. While most previous work has concentrated on the detection of flavonoids in biological samples by HPLC with electrochemical detection, some information can be found on their basic electrochemical properties. Preliminary cyclic voltammetry studies of flavonoids at a glassy carbon electrode have been described [1, 3]. Oxidation potentials in aqueous buffers ranged from 0.0 to 1.2 V depending on the pH of the solution and hydroxylation pattern of the flavonoid. Flavonoids with 3′,4′,5′-trihydroxy (pyrogallol-type) and 3′,4′-dihydroxy (catechol-type) substitution patterns were the most easily oxidized, while flavonoids with 1′,3′,5′-hydroxy (phloroglucinol-type) and phenol-type substitution patterns were much harder to oxidize. It was also observed that the oxidation of some flavonoids is chemically reversible while that of others is irreversible [1]. This property was used to distinguish catechol-containing flavonoids that showed reversible electrochemistry from flavonoids with phloroglucinol or pyrogallol-type hydroxylation where the electrochemistry was chemically irreversible [1].

This report describes the electrochemistry of four structurally related flavonoids: quercetin, quercitrin, rutin and luteolin. All of these compounds have the catechol group on the B-ring (3′,4′-dihydroxyl) in common and differ only by the substituent on C-3 (Fig. 1). The compounds were studied by a variety of electrochemical techniques to probe both the oxidation reaction and subsequent chemical transformations. Products were characterized by liquid chromatography.

Experimental

Materials

Quercetin and rutin were obtained from Sigma (St Louis, MO, USA). Galangin, 7-hydroxyflavone, 3-hydroxyflavone and chrysin

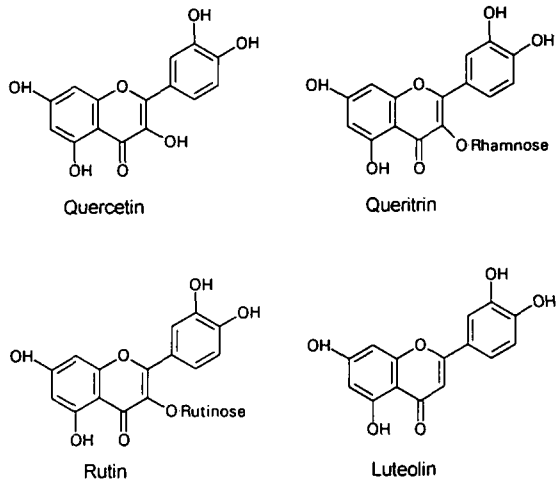

Figure 1
Structures for quercetin, quercitrin, rutin and luteolin.

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were purchased from Aldrich (Milwaukee, WI, USA). Quercitrin was purchased from Pfaltz and Bauer (Waterbury, CT, USA) and luteolin was obtained from Plantech UK (England, UK). All flavonoids were used as received without further purification. Acetonitrile was HPLC grade from Fisher. All other chemicals were reagent grade.

Apparatus

Cyclic voltammetry was carried out using a CV-27 voltammograph, a glassy carbon working electrode, a platinum auxiliary electrode, and a Ag/AgCl reference electrode (Bioanalytical Systems, Inc., West Lafayette, IN, USA). Rotating ring-disk voltammetry experiments were performed using an RDE-3 potentiostat, ASR2 analytical rotator, and glassy carbon/glassy carbon ring-disk electrode all from Pine Instruments Co. (Grove City, PA, USA).

A packed-bed bulk electrolysis flow cell described previously by Miner and Kissinger [6] was used to determine *n*-values and for the bulk electrolysis of quercetin. Vycor tubing was obtained from Bioanalytical Systems and activated prior to use. The vycor tubing was activated by boiling in a solution of 6 M nitric acid and 2% (v/v) 30% hydrogen peroxide for 30 min. The vycor was then washed with water prior to use. A Sage Model 355 syringe pump (Orion Research Inc., Boston, MA, USA) was used in these experiments at a flow rate of 1.3 ml min^{-1}.

Liquid chromatography was performed with a Shimadzu SCL-6A liquid chromatography system (Kyoto, Japan). Analytical separations were achieved using a Brownlee ODS, 5 μm particle size column (10 cm × 4.6 mm i.d.) and a 1.5 cm ODS guard column with a mobile phase of acetonitrile–ammonium phosphate (pH 2.5, 0.12 M; 15:85, v/v). A flow rate of 1.0 ml min^{-1} and an injection loop of 20 μl were used. Semi-preparative separation was achieved with a YMC-Pack ODS, 5 μm particle size column (30 cm × 10 mm i.d.) using a mobile phase of acetonitrile–ammonium phosphate (pH 2.5, 0.07 M; 30:70, v/v). A flow rate of 2.5 ml min^{-1} and an injection loop of 1 ml were used. Detection was carried out with either a Shimadzu SPD-6AV variable wavelength UV detector or a dual-electrode EG&G Model 400 electrochemical detector (Princeton Applied Research Princeton, NJ, USA) using glassy carbon electrodes and a Ag/AgCl reference electrode.

Cyclic voltammetry

A supporting electrolyte consisting of 0.05 M sodium phosphate buffer, 0.5 M potassium nitrate and 50% methanol was used in all electrochemical experiments. Solutions were carefully purged with argon prior to the electrochemical experiments. The scan rate was 200 mV s^{-1} unless otherwise indicated. The pH was varied from 1 to 10 in order to determine the effect of pH on the peak potential of the first anodic wave for quercetin, quercitrin, rutin and luteolin.

For the scan rate studies, the pH was 7.0 and the potential window was limited to the first anodic wave (peak I_a) and the corresponding cathodic wave (peak I_c) while the scan rate was varied from 10 to 1000 mV s^{-1}. A potential 50 mV past the peak potential observed with cyclic voltammetry was applied in all electrolysis experiments. The pH of these solutions was 4.0.

Rotating ring-disk voltammetry

Diffusion coefficients (D) were determined at an analyte concentration of 0.50 mM and a pH of 7.0. k_{obs}-Values were obtained using 0.1–2 mM analyte and pH values of 4.0, 7.0 and 9.0.

Calculations

The number of electrons transferred in the oxidation (n) was calculated from the electrolysis current determined from the flow cell using the Faraday equation written as:

$$n = \frac{60i}{fCF}, \qquad (1)$$

where n is the number of electrons, i is the measured current (amps), f is the flow rate (ml min^{-1}), C is the concentration (mol ml^{-1}), and F is the Faraday constant (96,400 coulomb/equivalent).

The linear relationship of the cyclic voltammetric peak potential (E_p) and pH was used to determine the number of protons transferred for a given oxidation through the Nernst equation as follows:

$$E = E^0 - \left(\frac{0.06}{n}\right)\log\left(\frac{[Ox][H^+]^a}{[Red]}\right) \qquad (2)$$

$$E = E^0 - \left(\frac{0.06}{n}\right)\log\left(\frac{[Ox]}{[Red]}\right) - 0.06\left(\frac{a}{n}\right)\log[H^+]. \qquad (3)$$

At E_p, [Ox]/[Red] is constant so that:

$$E_p = [E^\circ - k] + 0.06\left(\frac{a}{n}\right)\text{pH} \quad (4)$$

$$\text{slope} = 0.06\left(\frac{a}{n}\right), \quad (5)$$

where slope = the slope of E_p versus pH.

$$a = \text{slope}\left(\frac{n}{0.06}\right). \quad (6)$$

Therefore, a is the number of protons involved in the oxidation reaction. This approach is strictly true only for reversible systems, modifications for irreversible and quasi-reversible systems have been described in detail [7]. The uncertainties associated with the quasi-reversibility of the systems studied here do not lead to significant errors in the final results.

Results

Cyclic voltammetry

Cyclic voltammograms (CVs) of quercetin, quercitrin, rutin and luteolin at pH 7.0 are shown in Fig. 2. The first oxidation, I_a (E_p = 150 mV), corresponds to the oxidation of the 3′,4′-dihydroxy substituent on the B-ring of these compounds. The second oxidation, II_a (E_p = 500 mV), is due to the oxidation of the hydroxyl group at C-3. This oxidation is not observed for quercitrin, rutin or luteolin where the C-3 hydroxyl is either conjugated to a sugar or is absent. The final oxidation corresponds to the oxidation of the 5,7-dihydroxy substituent on the A-ring.

These conclusions were supported by comparing these peak potentials with those obtained for 3-hydroxyflavone, 7-hydroxyflavone, 5,7-dihydroxyflavone (chrysin) and 3,5,7-trihydroxyflavone (galangin; Fig. 3). None of these compounds has the 3′,4′-dihydroxy substitution and therefore do not exhibit the initial reversible oxidation wave. 3-Hydroxyflavone exhibits the oxidation wave at 500 mV while the oxidation of 7-hydroxyflavone occurs at higher potentials. This demonstrates the unique electrochemistry of the 3-OH group relative to hydroxylation at other positions. Assignment of the III_a oxidation wave to the 5,7-dihydroxy substitution is supported by the cyclic voltammetry of 5,7-dihydroxyflavone.

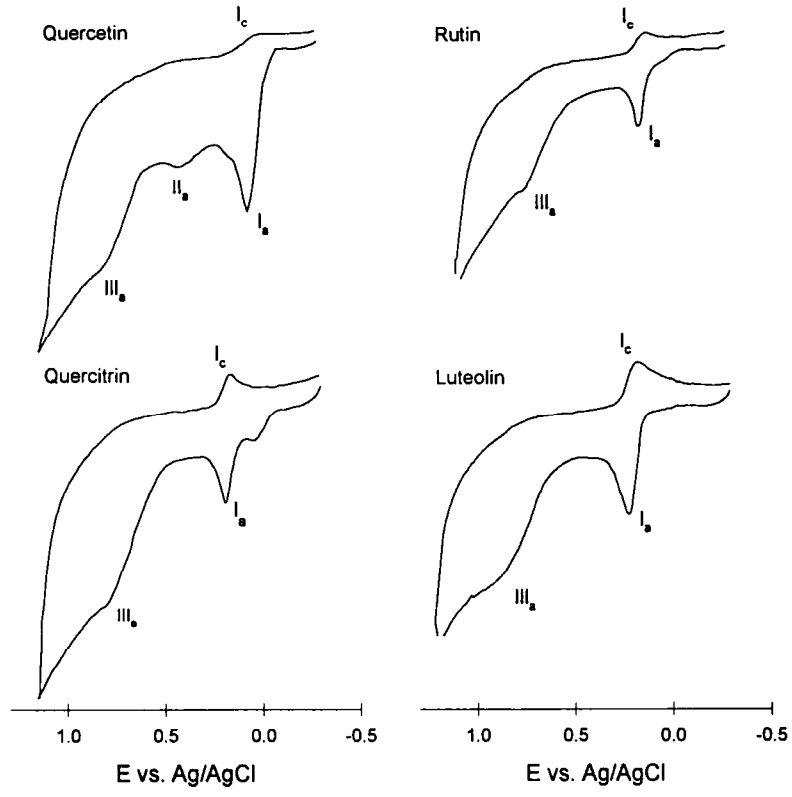

Figure 2
Cyclic voltammograms of quercetin, quercitrin, rutin and luteolin in 0.05 M sodium phosphate buffer (pH 7.0) at a scan rate of 200 mV s^{-1}.

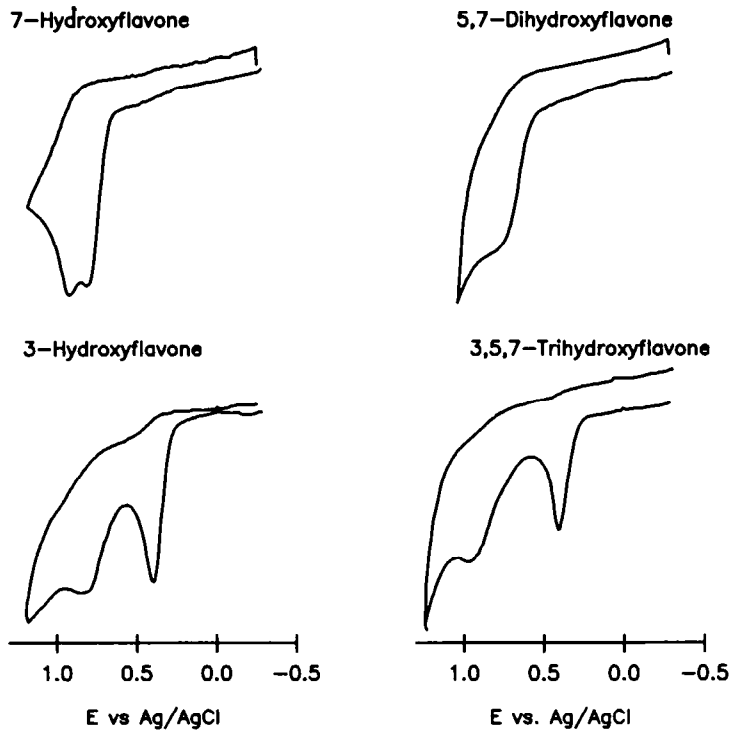

Figure 3
Cyclic voltammograms of 3-hydroxyflavone, 7-hydroxyflavone, 5,7-dihydroxyflavone (chrysin) and 3,5,7-trihydroxyflavone (galangin) in 0.05 M sodium phosphate buffer (pH 7.0) at a scan rate of 200 mV s^{-1}.

Table 1
Number of electrons, *n*-value, and protons, *a*, for the voltammetric oxidation peaks of quercetin, quercitrin, rutin and luteolin

Compound	Conc. (mM)	Peak	*n**	*a*/*n*†	*a*
Quercetin	4.89	I_a	2.2 ± 0.1	1.1	2.5
		II_a	3.6 ± 0.3		
		III_a	10.0 ± 0.4		
Quercitrin	3.36	I_a	2.1 ± 0.2	1.0	2.0
		II_a	2.4 ± 0.2		
		III_a	12.0 ± 0.5		
Rutin	3.36	I_a	1.9 ± 0.2	0.9	1.8
		II_a	2.2 ± 0.1		
		III_a	10.0 ± 0.4		
Luteolin	3.06	I_a	2.1 ± 0.1	1.0	2.0
		II_a	2.0 ± 0.2		
		III_a	7.1 ± 0.4		

*n-Values were obtained with a carbon packed bulk electrolysis flow cell in 0.1 M sodium phosphate (pH 4.0). The flow rate was 1.3 ml min^{-1}. Values are mean ±SD, n = 3.

†Obtained from the E_p versus pH curve from cyclic voltammetry performed in 0.05 *M* sodium phosphate buffer (μ = 0.5, KNO_3) at a glass carbon electrode. pH values of 2–10 were used. The scan rate was 200 mV s^{-1}.

The number of electrons transferred, n, was determined using the packed-bed bulk electrolysis flow cell. The number of protons involved in the initial oxidation was then determined from the slope of the E_p–pH curve. These results are summarized in Table 1. The initial oxidation of all of the 3′,4′-dihydroxy (catechol substituted) flavones is a $2e^-$-$2H^+$ chemically reversible process. The reversibility is highly scan rate dependent, indicating an electrochemical–chemical (EC) reaction mechanism. Only quercitin is further oxidized at wave II_a potentials. This oxidation also involves a two-electron step. The number of electrons transferred at wave III_a potentials was highly variable. This is due both to the complexity of

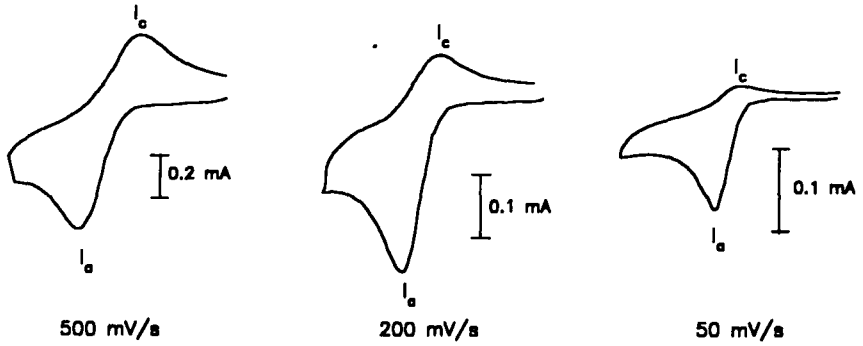

Figure 4
Cyclic voltammograms at a glassy carbon electrode of quercetin in 0.05 M sodium phosphate buffer (pH 7.0) at scan rates of 50, 200 and 500 mV s^{-1}.

the reactions and difficulty in measuring the faradaic current on the high background at this high potential.

The effect of scan rate on the reversibility of the I_a/I_c couple observed by cyclic voltammetry for 0.5 mM quercetin is shown in Fig. 4. As the scan rate increases the reversibility increases. This is consistent with a chemical rearrangement following the oxidation. The less time allowed for the rearrangement reaction (i.e. the faster the scan rate) the more oxidation product is available to be reduced. Comparison of the reversibility of the flavonoids at pH 7.0 is shown in Fig. 5. It is clear that the substituent at C-3 of these flavonoids greatly affects the reversibility of the I_c/I_a couple. The poorer the leaving group at this position, the slower the rearrangement kinetics of the electrochemically generated intermediate. The kinetics of this rearrangement were investigated using rotating ring-disk voltammetry.

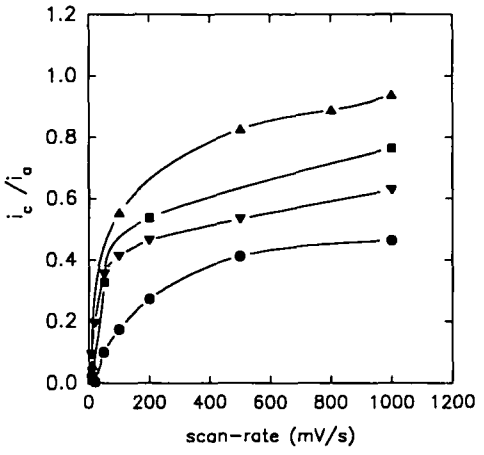

Figure 5
Cyclic voltammetric peak current ratios as a function of scan rate in 0.05 M sodium phosphate buffer (pH 7.0). Symbols: ●, quercetin; ▼, quercitrin; ■, rutin; and ▲, luteolin.

Rotating ring-disk voltammetry

Hydrodynamic voltammograms of the flavonoids were generated at a rotating disk electrode and the oxidation product was monitored cathodically at the ring electrode. The diffusion coefficients for the flavonoids were determined using the Levich equation and the *n*-values determined using the flow cell.

Observed pseudo-first-order rate constants (k_{obs}) for the disappearance of the oxidation product at peak I_a potentials were measured at the disk of the ring-disk electrode by the method of Bruckenstein and Feldman [8]; k_{obs} is determined from the slope of the plot of $\ln(N/N_o)$ versus $1/W$. N and N_o are the experimental current collection efficiencies for the test compound and that of a reversible system, respectively. N_o was determined using 0.50 mM hydroquinone at pH values of 5.0, 7.0 and 9.0. W is the rotation rate of the ring-disk electrode in rpm. An example working curve for the determination of k_{obs} is shown in Fig. 6 for 0.50 mM quercetin in pH 7.0 buffer; k_{obs} was calculated to be 0.07 ± 0.02 s^{-1} for quercetin at this concentration and pH. The variation of k_{obs} with pH in Fig. 7 reveals that the chemical reaction is base catalysed for all flavonoids studied. Quercetin exhibits the shortest half-lives, $t_{1/2}$ = 18.5 and 9.90 s, for the degradation of the electrochemically generated intermediate at pH values of 5.0 and 7.0, respectively. The anomaly seen at pH 9.0 for quercitin is most likely due to differences in pK_a between the 3-OH of quercetin and those of the C-3 substituents of quercitrin, rutin and luteolin. The pK_a of the 3-OH of quercitin is approximately 5 according to the literature compared with values >9 for most phenols [9].

The variation of k_{obs} versus concentration of

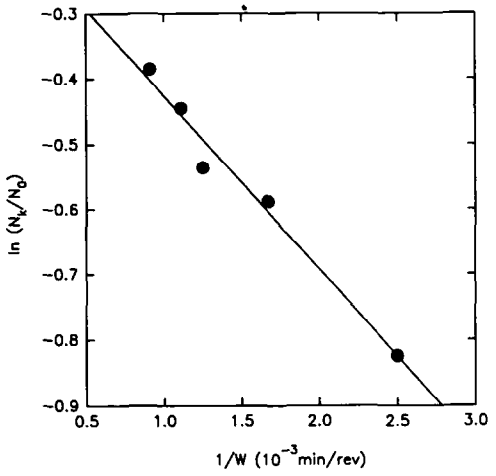

Figure 6
Working curve for the determination of k_{obs} by rotating ring-disk voltammetry for a 0.50 mM solution of quercetin in 0.05 M sodium phosphate buffer (pH 7.0). R_1 = 0.765 cm, R_2 = 0.833 cm and R_3 = 1.113 cm.

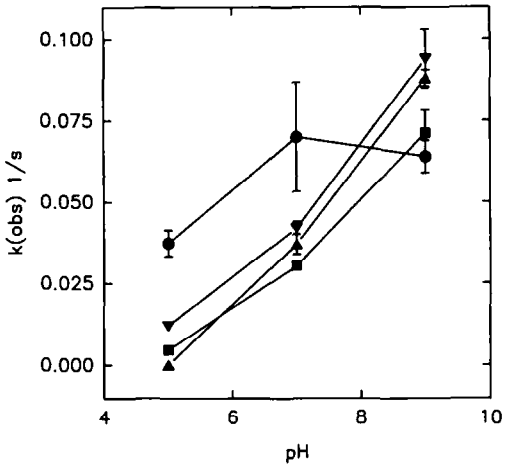

Figure 7
k_{obs} as a function of pH. Values are mean ±SD, n = 3–5 for 0.50 mM flavonoid solutions in 0.05 M sodium phosphate buffer. Symbols: ●, quercetin; ▼, quercitrin; ■, rutin; and ▲, luteolin.

the flavonoid at pH 7.0 showed that the kinetics for the reaction following the initial oxidation obeyed the general rate law

$$k_{obs} = k_0 + k_1[C], \qquad (7)$$

where k_{obs} is defined as above, and k_0 and k_1 are zero and first-order rate constants, respectively. The first-order rate constant was calculated from the slope of the k_{obs} versus concentration plot and the zero-order rate constant was calculated from the y-intercept of the plot. Table 2 lists the values for k_0 and k_1 obtained by rotating ring-disk voltammetry. The zero-order component of the rate law was dependent on the substituent at C-3. Quercetin exhibited the fastest kinetics while luteolin the slowest kinetics for the zero-order portion of the rate. The magnitude of k_0 descreased as the substituent at C-3 became a poorer leaving group. There was no correlation between the substituent at C-3 and k_1.

Table 2
Rate constants, k_0 and k_1, for the reactions following oxidation of quercetin, quercitrin, rutin and luteolin derived from rotating ring-disk voltammetry

Flavonoid	k_0 (1/s) × 10^2	k_1* (mM s^{-1}) × 10^2
Quercetin	4.7 ± 0.5	2.6 ± 0.2
Quercitrin	3.2 ± 0.5	1.5 ± 0.3
Rutin	2.0 ± 0.3	2.3 ± 0.4
Luteolin	2.0 ± 0.4	3.2 ± 0.9

* Error calculated from least-squares analysis.

Analysis of oxidation products

The flavonoids were oxidized in an electrolysis flow cell and the products characterized chromatographically. Oxidation in the flow cell results in 100% efficient electrolysis. Figure 8 shows a chromatogram of the flow cell products produced by the oxidation of quercetin at peak I_a potentials. These compounds were isolated using semi-preparative chromatography. The mixture of dilute isolated products was stable during this isolation period. Each fraction was characterized chromatographically, electrochemically and spectrographically. Comparison of the chromatographic retention times of product peak A and peak B with several reported enzymatic products of quercetin are listed in Table 3. On this basis, peak A was tentatively identified as phloroglucinol and peak B as protocatechuic acid. To confirm these assignments, chromatographically assisted hydrodynamic voltammograms (CAHDVs) were generated by making repetitive injections of the oxidation products and stepping the potential down in 50 mV increments until no analyte was detected. CAHDVs of the two oxidation products and caffeic acid are shown in Fig. 9 and the results are summarized in Table 3. These data indicate that the identities made on the basis of chromatographic retention are incorrect. In addition, because of the high oxidation potential and lack of reversibility neither of the isolated products contains the catechol group on the B-ring. UV–visible spectra of peaks A, B and quercetin were obtained by photodiode

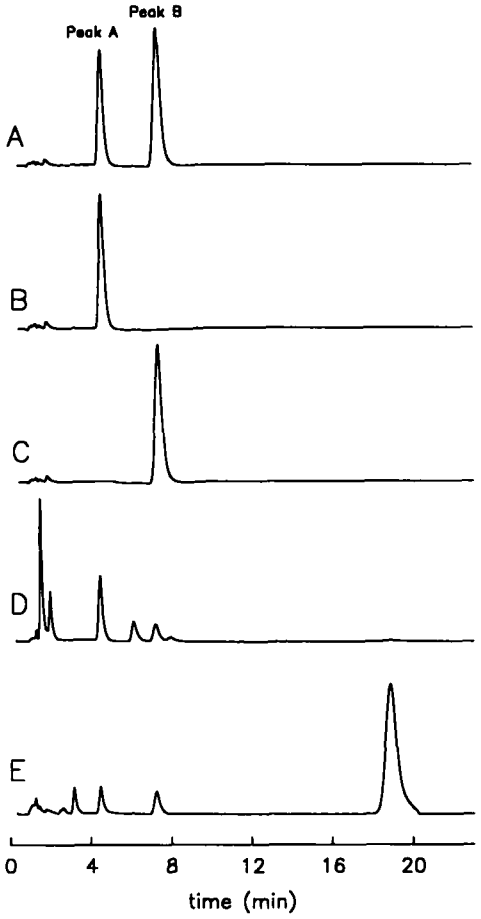

Figure 8
Chromatography of quercetin flow-cell oxidation products. A, direct injection of flow-cell products; B, isolated peak A; C, isolated peak B; D, isolated peak A following lyophilization; E, isolated peak B following lyophilization. Chromatographic conditions as described in the text.

array detection in 0.05 M ammonium phosphate buffer, pH 2.5 and 15% (v/v) acetonitrile and are shown in Fig. 10. The absorbance for quercetin at 350 nm is due to the conjugation

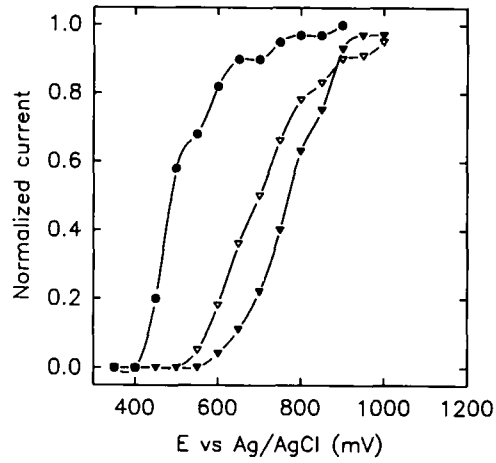

Figure 9
Chromatographically assisted hydrodynamic voltammograms (CAHDV) of quercetin oxidation products and caffeic acid.

between the ketone of the C-ring and B-ring [10]. Since the absorbance at 350 nm is absent, this conjugation must be lost in both of the oxidation products.

The isolated peaks A and B were then lyophilized for further spectroscopic analysis. This resulted in the decomposition of these compounds as illustrated in Fig. 8. Other attempts to isolate these compounds for NMR and IR analysis resulted in similar decomposition. This unfortunately leaves us with only the ability to compare characteristics to known compounds and not the ability to directly determine the structure of the oxidation products.

Discussion

The initial oxidation at a glassy carbon

Table 3
Characterization of quercetin oxidation products

Compound	Retention time (min)	Reversible (collection efficiency)
Phloroglucinol	3.0	0.24
Protocatechuic acid	6.8	0.49
Caffeic acid	17.8	0.45
2,4,6-Trihydroxybenzoic acid	3.8	0.21
Peak A	3.2	ND
Peak B	6.6	ND
Peak C (quercetin)	23.8	0.40

*Reversibility determined from dual-electrode amperometric detection in the series configuration with liquid chromatography. The number is the ratio of the downstream cathodic current to the upstream anodic current. ND is no downstream response detected (system is irreversible).

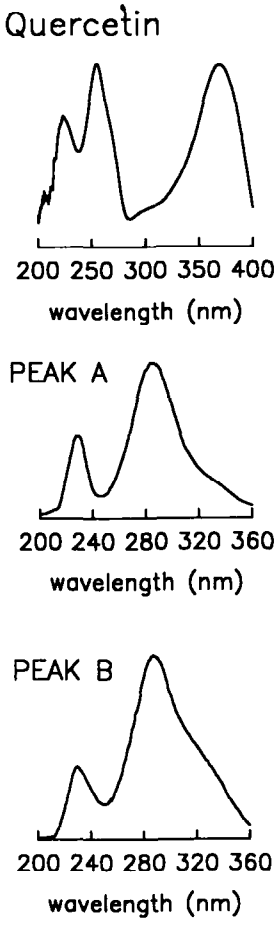

Figure 10
UV–visible spectra of quercetin, peak A and peak B in 0.05 M ammonium phosphate (pH 2.5) with 15% (v/v) acetonitrile.

electrode of quercetin, quercitrin, rutin and luteolin is a $2e^{-}$-$2H^{+}$ process giving an *o*-quinone species. This *o*-quinone then undergoes homogeneous rearrangement by at least two processes: one zero-order and the other pseudo-first-order. The zero-order process is strongly dependent on the leaving group attached at C-3. Luteolin is unsubstituted at this position and displayed reversibility approaching that of catechol. Liquid chromatographic analysis with electrochemical and photodiode array detection revealed two oxidation products for quercetin. The electronic structure of these compounds is significantly different from that of quercetin.

The zero-order process is likely an intramolecular rearrangement involving the substituent at C-3. A possible mechanism is shown in Fig. 11. The catechol group is oxidized to the quinone which undergoes intramolecular rearrangement followed by keto–enol tautomerization. This mechanism fits nicely with the available kinetic data, since there is a strong dependence on the substituent at C-3. Quercetin with a proton attached at C-3 exhibits the fastest zero-order kinetics. Rutin with a disaccharide attached to this oxygen exhibits the slowest zero-order kinetics. Luteolin cannot react by this mechanism since it is unsubstituted at C-3 and consequently exhibits the highest degree of reversibility.

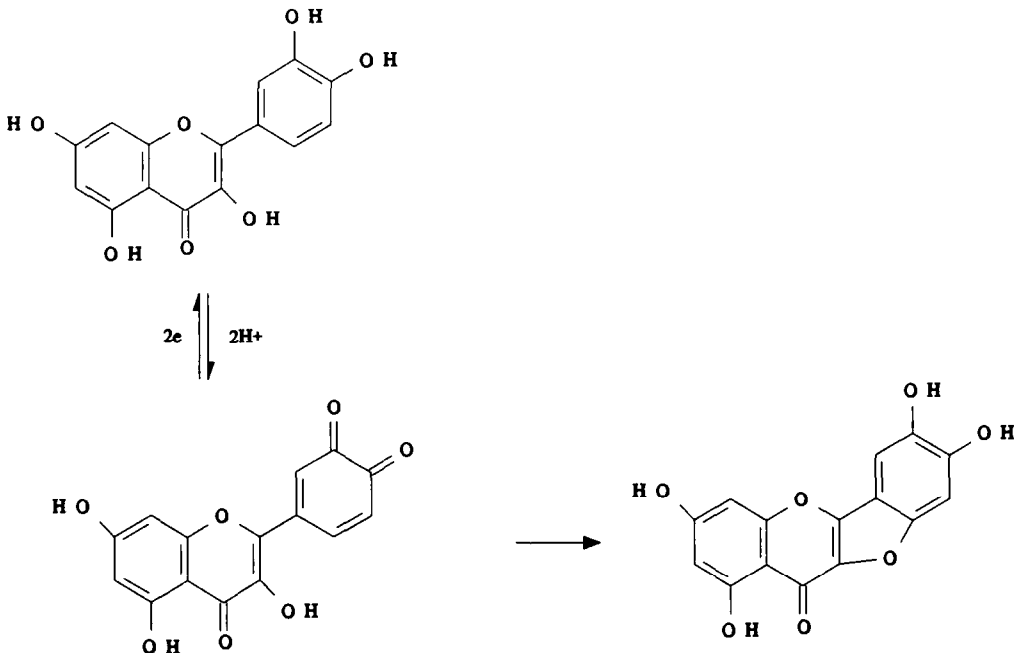

Figure 11
Proposed mechanism for the zero-order degradation of the electrochemically generated oxidation product of quercetin.

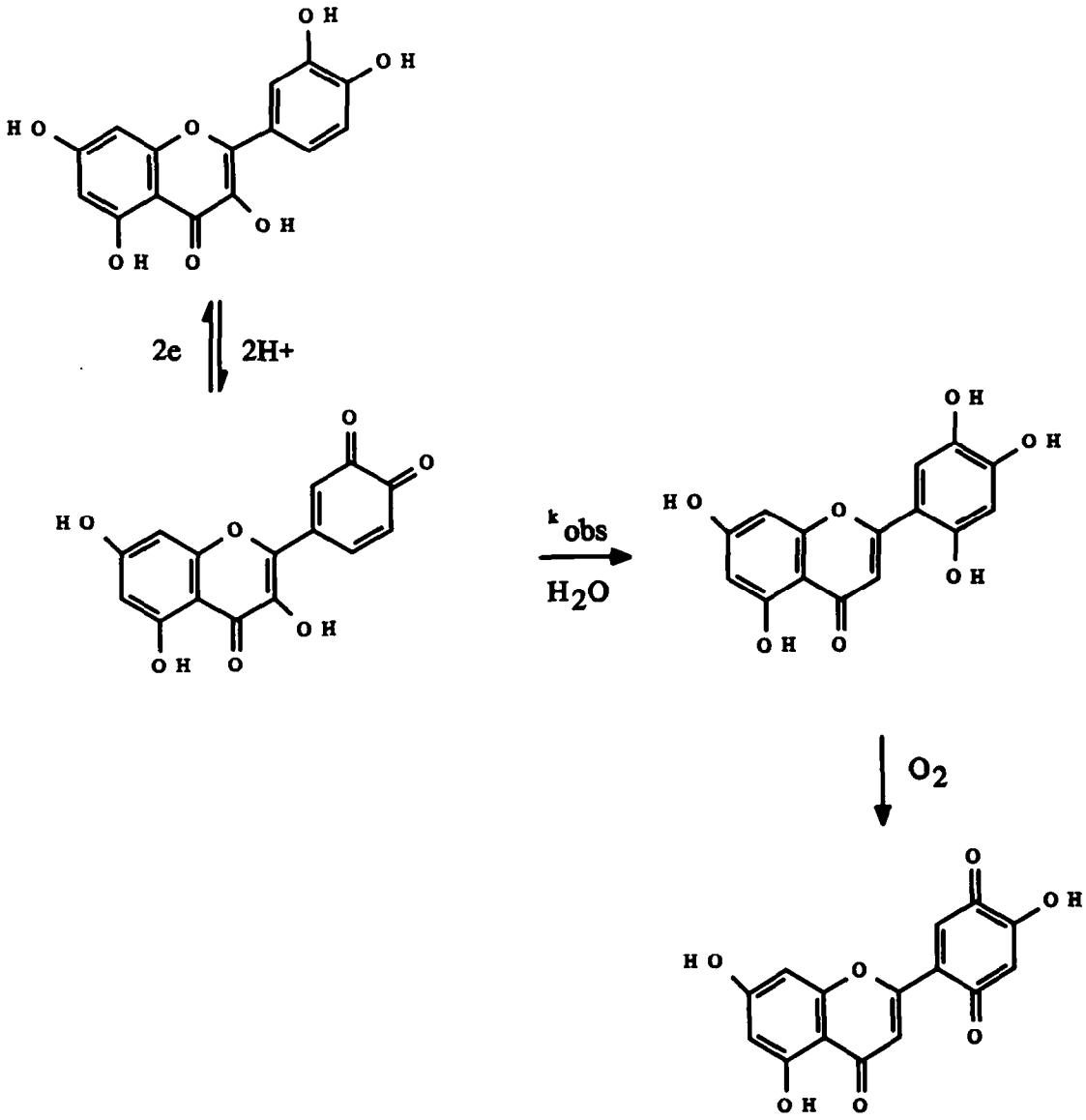

Figure 12
Proposed mechanism for the first-order degradation of the electrochemically generated oxidation product of quercetin.

A possible mechanism for the pseudo-first-order process is shown in Fig. 12. This mechanism involves nucleophilic attack of H_2O at C-6′ forming the 3′,4′,6′-trihydroxylated derivative. Because of the oxygen in the C-ring the molecule would not be planar and the carbonyl at C-6′ would no longer be conjugated with the B-ring. These type of quinones are unstable and subject to nucleophilic attack which leads to the formation of dimers and trimers. Confirmation of these mechanisms awaits development of techniques to isolate the products of the oxidation in a manner allowing structural identification.

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