ANTIOXIDANTS & REDOX SIGNALING
Volume 3, Number 6, 2001
Mary Ann Liebert, Inc.

Forum Method Communication

Electrochemical Detection of Natural Antioxidants: Principles and Protocols

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ABSTRACT

An overview is provided of the use of cyclic voltammetry and electrochemical detectors for HPLC to characterize antioxidants that are active as reducing agents at inert electrodes. Results are presented for cyclic voltammetry at a glassy carbon electrode in 50% methanol, 50% 0.1 *M* $HClO_4$, a solution typically used in HPLC separations of natural antioxidants. The relative reducing strength of each antioxidant is estimated by the formal potential, and information is also obtained regarding the reversibility of the oxidation of the antioxidants, and the extent to which the carbon electrode is contaminated by the products of oxidation. Cyclic voltammetry of complex mixtures such as blood serum and wine produces a measure of the total antioxidant status due to antioxidants with a low oxidation potential. The results of cyclic voltammetry studies are relevant to interpreting the performance of carbon electrodes in electrochemical detectors for HPLC. Antioxid. Redox Signal. 3, 941–955.

INTRODUCTION

In considering electrochemical methods for characterizing antioxidants in biological systems, we are seeking to match the redox activity of a chemical in solution with redox activity at an inert electrode. Likewise a link is sought between the strength of an antioxidant in a cellular environment and the potential at which it itself is oxidized at an electrode (the more powerful reducing agent will have a less positive oxidation potential). The fact that an antioxidant is a reductant in its interaction with radicals generally means that it can be oxidized at an electrode, but this is not an absolute relation. Just as different antioxidants react with different free radicals and oxidants to varying extents, so too they will be electroactive to varying degrees on different electrode surfaces. Despite these variations, the rank established at an electrode will provide an indication of the strength of an antioxidant as a reducing agent in solution, and will produce details specific to that antioxidant that allow identification and quantification. On the other hand, antioxidants that inhibit the formation and activity of reactive oxygen and nitrogen species by other modes of operation, such as by metal chelation or as enzymes, are not often monitored by an electrochemical method.

The main focus of this overview will be cyclic voltammetry, in which each antioxidant is characterized by the current–potential relationships exhibited at an inert electrode. A related application of electrochemistry, which will be introduced first, involves monitoring antioxidants separated by high-performance liquid chromatography (HPLC) at an electro-

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chemical detector (ECD), where the concentration of the antioxidant is determined by the current produced. The strengths and weaknesses of these applications of electrochemistry to the study of antioxidants in biological systems will be considered below.

HPLC-ECD

There has been considerable progress in the area of electroanalytical chemistry involving organic compounds since the development of reliable potentiostats and the ability to precisely control and measure potential (E) and current (I) as a function of time (41). The key act of electron transfer occurs at the boundary where the (working) electrode makes contact with reactive species in a conducting solution. The rate of electron transfer, measured as the size of the current that flows, is determined by the speed of the reaction at the applied potential; it can also be dependent upon the rate at which the reactive species are brought to the electrode surface, by diffusion and convection, or at which products are removed. Two further electrodes are usually required to complete the electrochemical cell: a counter electrode through which the current also flows as part of the electrical circuit, and a reference electrode to define a scale of potential at the working electrode, but which receives only an infinitesimal current flow.

If working electrodes made of materials such as gold, platinum, amalgams of these metals with mercury, or glassy carbon are maintained at a constant potential, we have the ability to detect currents due to species that can be oxidized or reduced at that potential, in a method known as amperometric detection. This is the basis of ECDs used in conjunction with the separation afforded by HPLC (30). Glassy carbon has been found to be the most suitable for use with organic molecules. It is generally resistant to solvents, unlike most metals, and shows low background currents, as will be illustrated in cyclic voltammograms below.

A range of compounds, relevant to the study of antioxidants and their role in limiting oxidative damage *in vivo*, have been detected using HPLC-ECD, as recently reviewed by Acworth *et al*. (1). These include indirect approaches to monitor levels of the highly reactive hydroxyl radicals using spin traps such as salicylic acid to form dihydroxybenzoic acids. Levels of citrulline have been used to quantify the reactive nitric oxide radical, which can also be monitored directly using modified electrodes. A number of molecules can also be detected by HPLC-ECD that serve as markers of damage caused by reactive oxygen and nitrogen species. These include products formed by the oxidation of aromatic amino acids such as dityrosine and 3-nitrotyrosine, oxidized DNA bases (8), and products of lipid peroxidation such as 4-hydroxynonenal. By their very nature, antioxidants produce a good response at ECDs. Species such as ascorbic acid, uric acid, cysteine (10), glutathione, melatonin, glutamate, tocopherols, ubiquinones, carotenoids (14), retinoids, lipoic acid, homocysteine (1), polyphenolic antioxidants (6, 28, 29, 38), and various crude drugs (32) have been monitored regularly in biological media by using HPLC-ECD. The ECD method displays extremely low detection limits for electroactive species into the picomole level, frequently 10–50 times more sensitive than UV-visible detectors (see the articles referred to above).

To establish the optimum electrode potential in ECD, for quantifying the above species, it is often necessary to establish a hydrodynamic voltammetry plot (4). In this case, repeated HPLC injections are made of the substance of interest with the detector set at progressively higher electrode potentials. The optimum potential is the one at which the current due to the analyte is at a maximum, whereas the background current from the solvent and other interferants is at a minimum. Compounds with the same chemical characteristics give similar responses, but at times the response can vary considerably for a small change in structure (*e.g.*, having two −OH groups on a benzene ring in the *meta* rather than the *ortho* position can shift the oxidation potential by several hundred millivolts). Differences in the redox properties of analytes allow them to be distinguished from quite complex mixtures using hydrodynamic plots. As we will see below, more detailed information of this sort is obtained by cycling the potential at an electrode in a solution of the analyte.

A well known problem with exposing electrodes to biological media is contamination of the electrode surface with lipids, proteins, and other surface-active materials. This difficulty can be overcome through the use of permeable membranes on electrochemical sensors, and by the separation afforded by the HPLC column. However, the target antioxidants, or their oxidation products, can irreversibly adsorb on the electrode surface, significantly altering the current response of the electrode, and illustrating the need to clean the electrode between measurements. This point will also be illustrated below through the behavior of standard solutions of antioxidants at a glassy carbon electrode. One approach to overcome this problem when using gold or platinum electrodes is by pulsing the electrode to a large positive potential to fully oxidize and remove the adsorbates, and then applying a negative potential to remove the surface oxide, before returning to the measurement potential—the whole cycle may take <1 s. The actual reading is usually taken once the charging current, which follows any step in the potential, has declined, and often for a period of 16.7 ms (one 60-Hz waveform). This technique is known as pulsed amperometric detection (21, 22), and has allowed many compounds to be analyzed that were thought to be electroinactive. Compounds such as aliphatic amines and alcohols, which are so reactive that they rapidly contaminate the electrode surface, are now effectively detected at a freshly regenerated surface. Among the metal electrodes, gold tends to be used more often than platinum, due to the greater cathodic response seen at platinum to dissolved oxygen, which can be minimized at gold. In other cases in which maximum sensitivity is required, platinum can be more effective.

A range of designs for HPLC-ECD are available, including multiple electrodes to allow detection at different electrode potentials. A leading approach makes use of a range of coulometric electrodes in series, which allow total oxidation or reduction of all the species that can react at a particular potential (1). By measuring the total charge passed, improved sensitivity is achieved, which is of particular interest in the detection of oxidation products from biological samples at low concentrations. By using a flow-through design, an antioxidant will react completely at the first electrode of sufficiently positive potential, allowing the voltammetric profile to be determined with a single experiment. If species coelute, their separate responses can be detected on the coulometric array, which is not possible with amperometric electrodes.

As well as measuring the levels of specific antioxidants, or the products of oxidative damage, the sum total of the principal antioxidants seen in HPLC-ECD, or the proportion of oxidized and reduced forms, can be used as a measure of the antioxidant activity of the medium or the health of the cellular tissue. These measures stand alongside others based on the ability of the solution to react with free radicals as a means of quantifying the total antioxidant status of the fluid.

Besides HPLC-ECD, the electrochemical technique of cyclic voltammetry has been applied to characterize the reducing strength of antioxidants, and their reversibility. This information is in turn relevant to the performance of electrodes in ECDs for HPLC. The direct measurement of the redox potential, as an indication of the reducing strength of a solution, is not considered here, owing to its variable response to natural antioxidants (24). The following results illustrate the application of cyclic voltammetry to characterize antioxidants using a solution typical of HPLC mobile phases used for the separation of phenolic antioxidants.

MATERIALS AND METHODS

The following analytical grade antioxidants were used: L-ascorbic acid (Scientific Supplies), quercetin (Sigma Q-0125), epicatechin (Sigma E-1753), morin (May & Baker), catechin (Sigma C-1251), caffeic acid (Sigma C-0625), gallic acid (Riedel–de Haën), rutin hydrate (Sigma R-5143), butylated hydroxytoluene (BHT) (Sigma B-1378), resveratrol (Sigma R-5010), uric acid (BDH), ferulic acid (Sigma F-3500), vanillic acid (Sigma V-2250), *p*-coumaric acid (Sigma C-9008), and 3,5-dihydroxybenzoic acid (Aldrich D11,000). HPLC grade methanol (Lab-Scan)

and Milli-Q water were used to make up the solutions.

Cyclic voltammograms were recorded using Bioanalytical Systems (BAS) equipment at a 100A electrochemical analyzer with BAS RDE-1 rotating disk electrode. The working electrode was a 3-mm glassy carbon disk electrode (MF-2066), which was cleaned by polishing using 3-μm alumina powder (PK-4 polishing kit) between runs; 3-mm gold (MF-2068) and platinum (MF-2067) electrodes were also used at times. A Ag/AgCl reference electrode (RE-5B; +207 mV versus *she*) was used in conjunction with a platinum counter electrode (MW-1033) in a low-volume cell (MR-3760).

Solutions of antioxidants, to a final concentration of 0.5 m*M*, were dissolved in 25 ml of methanol and added to 25 ml of 0.1 *M* $HClO_4$, which gave a measured pH of 1.40 ($\pm$0.05). About 20 ml was added to the cell and N_2 gas was bubbled through the solution to remove O_2. An initial cyclic voltammogram was recorded soon after the glassy carbon electrode was inserted into the solution, to minimize adsorption of the antioxidant onto the electrode surface prior to the first run. The scan rate was generally 100 mV s^{-1}, and the initial scan was taken from -100 mV to 100–150 mV past the first anodic peak.

Cyclic voltammograms of a Sauvignon Blanc white wine (Esk Valley, New Zealand, 1999, pH 3.51) were taken with the glassy carbon electrode directly in the wine, whereas a Pinot Noir red wine (Coopers Creek, New Zealand, 1998, pH 3.60) was diluted 20 times in a model wine solution, consisting of 12% ethanol, 0.05 *M* L-tartaric acid (Panreac 141066), and added NaOH to give a pH of 3.6.

Fifty-milliliter samples of blood were taken from the antecubital fossa of one healthy male subject and transferred immediately to eight 10-ml red top vacutainers (clot activator tubes, Becton Dickinson). They were allowed to stand for 1 h at room temperature and then were spun at room temperature for 15 min at 4,000 rpm. The supernatant was collected and pooled (~20 ml). Cyclic voltammograms of the blood serum were recorded at the freshly abraded glassy carbon electrode.

RESULTS AND DISCUSSION

Cyclic voltammetry is used widely in electrochemistry to characterize electroactive molecules and electrode surfaces. In this technique, the potential is scanned at a controlled rate, and the current produced by oxidations or reductions is recorded continuously. In Fig. 1, cyclic voltammograms at clean platinum, gold, and glassy carbon electrodes are recorded using the supporting electrolyte chosen in this study as one typical of mobile phases used in HPLC separations of antioxidants. This electrolyte comprised 50% methanol, in which most phenolic antioxidants are soluble, and 50% aqueous 0.1 *M* $HClO_4$ to lower the pH; the perchlorate ion also provides for electrical conductivity between the electrodes, but is itself electroinactive and will not produce an additional current response.

There was significant anodic (positive) current during the cyclic voltammogram at the platinum electrode beyond 200 mV due to oxidation of methanol; at potentials greater than ~1,000 mV, oxygen is generated by the oxidation of water. The fact that the oxidation current decreases on the forward scan beyond 750 mV is due largely to the formation of a resistive oxide on the platinum surface. Conversely on the reverse scan, a large increase was seen in the current at potentials less than 650 mV,

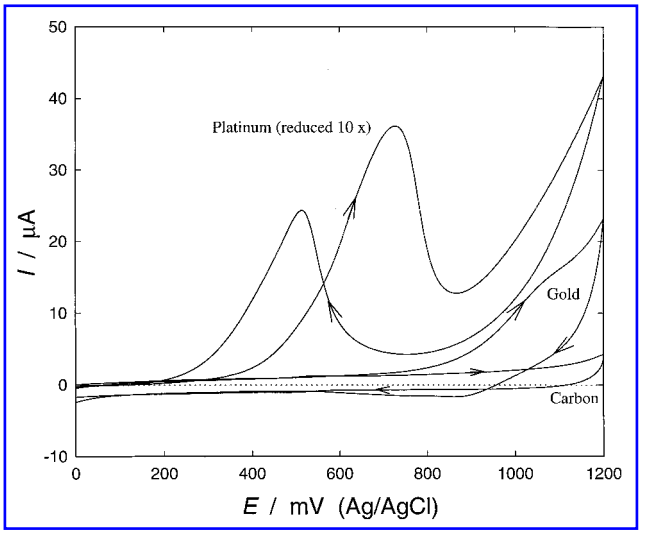


FIG. 1. Cyclic voltammograms taken at 3-mm diameter platinum, gold, and glassy carbon electrodes at 100 mV s^{-1} in 50% 0.1 *M* $HClO_4$ and 50% methanol (pH 1.4). The arrows indicate the direction of each potential scan.

despite the decrease in the oxidative power of the electrode, due to the removal of the platinum oxide and reestablishment of the more active bare platinum surface. Gold was much less active than platinum for the oxidation of methanol, but a rise in current was still seen from ~700 mV, which would interfere with the detection of other compounds.

The situation with a glassy carbon electrode in the same electrolyte was quite different. As seen in Fig. 1, only a very small background current was generated, which is due to a charging current as the potential is scanned and some small oxidation and reduction of the solvent. Glassy carbon is thus an excellent electrode material to study the electrochemistry of natural antioxidants.

If we now add an antioxidant to the solution, and record the cyclic voltammogram at the carbon electrode, a set of anodic and cathodic (negative current) peaks are obtained. The result for a solution of 0.5 m*M* caffeic acid is shown in Fig. 2. As with many phenolic antioxidants, the reaction has the following form:

$$R \rightleftharpoons O + 2H^+ + 2e^- \qquad (1)$$

where R represents the reductant (antioxidant) and O the product of oxidation, itself an oxidant. The reaction with caffeic acid (structure given in Chart 1) involves an *ortho*-diphenol moiety reacting to an *ortho*-quinone, a feature typical of the antioxidants that react in the 500–600 mV region:

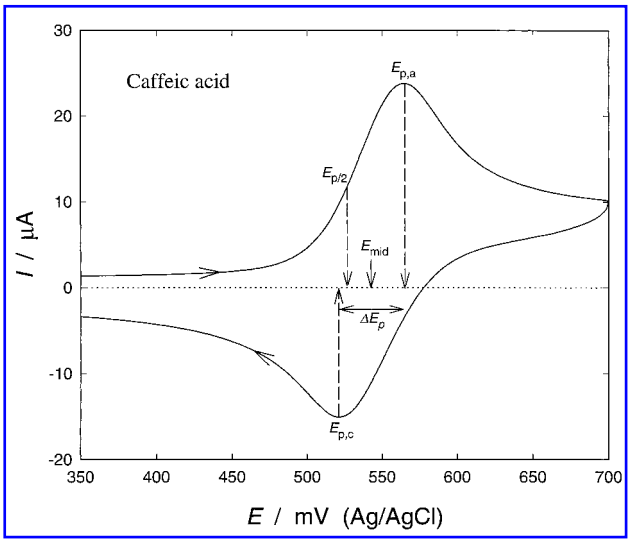


FIG. 2. Cyclic voltammogram of 0.5 m*M* caffeic acid in 50% 0.1 *M* $HClO_4$ and 50% methanol taken at a 3-mm diameter glassy carbon electrode at 100 mV s^{-1}. $E_{p,a}$ and $E_{p,c}$ are the anodic and cathodic peak potentials, respectively; $E_{p/2}$ is the half-peak potential of the oxidation wave; ΔE_p is the peak separation; and E_{mid} is the potential midway between $E_{p,a}$ and $E_{p,c}$.

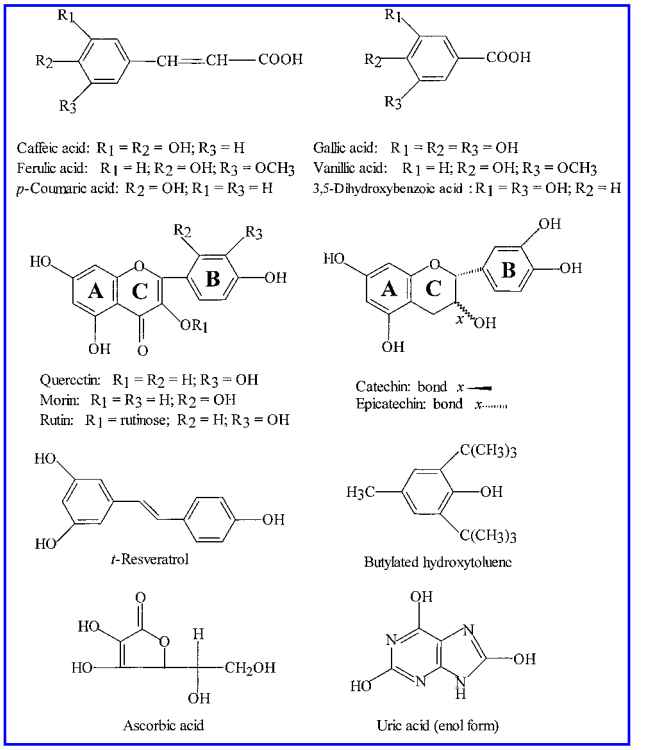
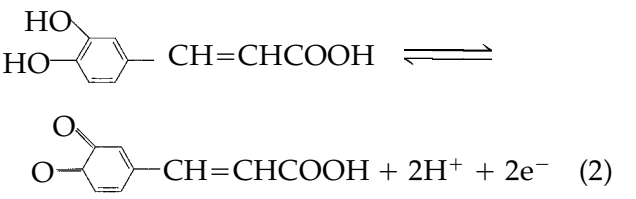


CHART 1. Structures of the antioxidants examined in this report.



$$CH{=}CHCOOH + 2H^+ + 2e^- \qquad (2)$$

The reversibility of the reaction at the carbon electrode will vary with different antioxidants, and O may be susceptible to further chemical reactions. The concentration dependence of the potential (E in mV) for this reaction will also vary with pH according to the Nernst equation ($n = 2$):

$$E = E^o - \frac{RT}{nF}\ln\frac{(R)}{(O)(H^+)^2} = E^o - 59\ \text{pH} - \frac{59}{2}\log\frac{(R)}{(O)} = E^{o\prime} - 29\log\frac{[R]}{[O]} \qquad (3)$$

where (R) and (O) are the activities, and [R] and [O] the concentrations, of the reduced and oxidized forms, respectively, n is the number of electrons passed in the reaction (here $n = 2$), $R = 8.314\ \text{J mol}^{-1}\ \text{K}^{-1}$, $T = 298\ \text{K}$, $F = 96{,}487\ \text{C mol}^{-1}$, and E^{o} is the standard potential when the activity of all species is 1 mol L^{-1}. $E^{o\prime}$ is the formal potential, which compensates for the particular pH and ionic strength of the electrolyte being used, but still represents the potential at which [R] = [O], and which we want to measure to quantify the reducing power of each antioxidant.

On the forward scan (in Fig. 2), an increase in current begins from ~ 450 mV as the potential approaches $E^{o\prime}$, and climbs exponentially. However, the rapid rise in current does not last for long, and a peak is seen ($E_{p,a}$) at 564 mV and the current decreases. This decrease in current is not due to some change at the electrode surface, but is a natural consequence of the depletion of caffeic acid near the electrode surface and the emergence of a situation in which the current is limited by the diffusion of caffeic acid from the bulk solution (3). The size of the current at the peak ($I_{p,a}$) is a function of a number of variables, including the electrode area (A), scan rate (v), bulk concentration of the antioxidant (C_R^*), and the diffusion coefficient (D_o):

$$I_{p,a} = (2.69 \times 10^5) n^{3/2} A D_o^{1/2} v^{1/2} C_R^* \qquad (4)$$

From the cyclic voltammogram for caffeic acid, we can determine a number of values of interest, including the anodic peak potential ($E_{p,a}$) and the potential at which the current reaches half its maximum ($E_{p/2}$), the half-peak potential. For a reversible (Nernstian) wave, in which the oxidation and reduction occur easily and rapidly at the electrode, and the formation of the peak is purely under diffusion control, the difference $|E_{p,a} - E_{p/2}|$ is $56.5/n$ mV at 25°C, or in the case of caffeic acid ($n = 2$) $\sim$28 mV. The value of 37 mV obtained for caffeic acid (Table 1) shows good, although not perfect, reversibility for this antioxidant. The formal potential $E^{o\prime}$ is expected to lie about half

Table 1. Oxidation and Reduction Potentials of 0.5 m*M* Antioxidants Measured at a 3-mm Glassy Carbon Electrode at 100 mV s^{-1} in 50% 0.1 *M* $HClO_4$ and 50% Methanol (pH 1.4)

E *in mV (Ag/AgCl)*	$E_{p/2}$	$E_{p,a}$	$E_{p,c}$	ΔE_p	$E_{p,a} - E_{p/2}$	E_{mid}	$\frac{(E_{p,a} + E_{p/2})}{2}$	$I_{p,a}$ (μA)
Ascorbc acid	356	446	—	—	90	—	401	11.7
Quercetin	484	526	480	46	42	503	505	20.6
	(726)	790						14.7
	(916)	966						14.2
Epicatechin	490	531	476	55	41	502	511	17.3
	(839)	932						23.7
Morin	495	535	—	—	40	—	515	18.0
	(1,073)	1,253						31.7
Catechin	511	567	464	103	56	516	539	14.2
	(854)	939						19.5
Caffeic acid	527	564	522	42	37	543	546	23.9
Gallic acid	532	568	—	—	36	—	550	24.3
	(857)	930						23.0
Rutin	568	606	569	37	38	588	587	20.5
	(1,217)	1,280						32.3
BHT	(566)	(677)	—	—	(111)	—	(622)	7.0
	(811)	902						14.8
trans-Resveratrol	596	710	—	—	114	—	653	11.6
	(942)	1,032						23.4
Uric acid	646	672	—	—	26	—	659	10.4
Ferulic acid	688	791	—	—	103	—	740	23.7
Vanillic acid	879	923	—	—	44	—	901	23.0
p-Coumaric acid	864	955	—	—	91	—	910	18.3
3,5-Dihydroxybenzoic acid	994	1,078	—	—	84	—	1,036	23.6

The values in parentheses are estimates. In places where no values are given, a cathodic peak was not seen and the oxidation was largely irreversible.

way between $E_{p/2}$ and $E_{p,a}$; the value of $(E_{p,a} + E_{p/2})/2$ for each antioxidant has also been included in Table 1. This also reminds us that $E_{p/2}$ is different from the half-wave potential $E_{1/2}$, reported in polarography at a continuously renewed dropping mercury electrode, which is used as a direct approximation for $E^{o\prime}$.

On the reverse scan, the product of caffeic acid oxidation (O), which has yet to diffuse far away from the electrode surface and is practically absent from the bulk solution, can be reduced as the electrode potential is lowered toward $E^{o\prime}$. Once again, as the levels of O near the electrode become depleted, a cathodic peak ($E_{p,c}$) is seen as the reaction comes under diffusion control. The current at the cathodic peak ($I_{p,c}$) is less intense than the anodic peak current ($I_{p,a}$) due to the continued oxidation of R in the background and the more limited availability of O from the bulk solution. For a reversible (Nernstian) system, the difference $\Delta E_p = |E_{p,a} - E_{p,c}|$ is close to $59/n$ mV at 25°C (being a little smaller as the potential at which the scan is reversed is increased), or 28–30 mV for antioxidants with $n = 2$. The value of 42 mV observed for caffeic acid (Table 1) is a further indicator that caffeic acid displays reasonably good reversibility at the glassy carbon electrode.

Finally, the potential (E_{mid}) midway between $E_{p,a}$ and $E_{p,c}$ can also be used to approximate the formal potential associated with R and O, and will provide a better estimation when the anodic peak is very broad. For caffeic acid, a value of 543 mV was obtained, close to the value of $(E_{p,a} + E_{p/2})/2 = 546$ mV. However, as a cathodic peak is not seen with several antioxidants, $(E_{p,a} + E_{p/2})/2$ has been used to rank the relative reducing strengths of the antioxidants in Table 1.

A further type of experiment using cyclic voltammetry that shows how the processes are under diffusion control is to use a rotating disc electrode. As the electrode rotates, it draws solution up toward the electrode surface and supplies a steady flow of antioxidant. Once a sufficiently positive potential has been applied, a limiting current will be produced, which, according to the Levich equation, is proportional to the square root of the rotation rate ($\omega^{1/2}$), and directly proportional to the concentration of the antioxidant in the bulk solution.

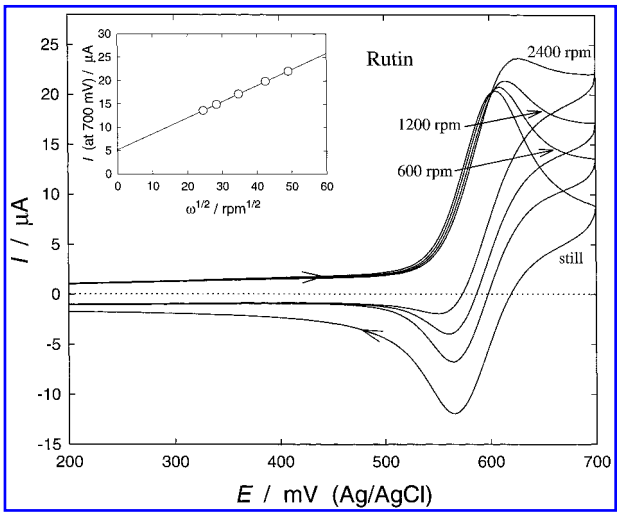


FIG. 3. Cyclic voltammogram of 0.5 m*M* rutin in 50% 0.1 *M* $HClO_4$ and 50% methanol taken at a 3-mm diameter glassy carbon electrode at 100 mV s^{-1} and at a range of electrode rotation rates from still to 2,400 rpm. The **inset** gives the current at 700 mV as a function of the square root of the rotation rate ($\omega^{1/2}$).

In Fig. 3, cyclic voltammograms for 0.5 m*M* rutin are shown at a range of electrode rotation rates. We can note from the cyclic voltammogram taken under still conditions that $(E_{p,a} + E_{p/2})/2$ for rutin at 587 mV was 41 mV more positive than, and ΔE_p at 37 mV was similar to, that for caffeic acid (Table 1). These results show that rutin is more difficult to oxidize and is a weaker reducing agent, while showing a similar degree of reversibility as caffeic acid. As the rotation rate is increased, the current falls off less markedly after $E_{p,a}$ is reached, and by 2,400 rpm a plateau is nearly formed. The limiting current at 700 mV is directly proportional to $\omega^{1/2}$ (see the inset of Fig. 3), but has a nonzero intercept when extrapolating to zero rotation rate, probably associated with oxidation of the solvent. It is also likely that oxidation products may be adhering to the electrode surface, lowering the efficiency with which further rutin is oxidized. A further effect of electrode rotation is to rapidly remove soluble reaction products that are no longer available for reduction on the reverse scan, hence a progressively smaller cathodic peak is seen at higher rotation rates.

Returning to the unrotated electrode, the effects of electrode contamination are readily seen in the case of epicatechin (Fig. 4). Epicat-

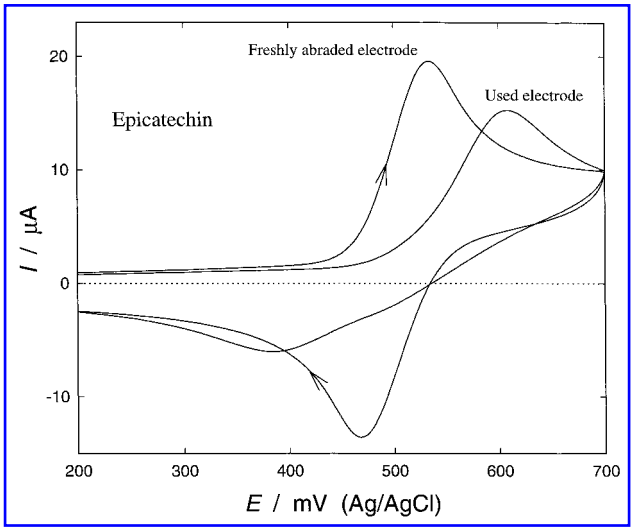


FIG. 4. Cyclic voltammogram of 0.5 m*M* epicatechin in 50% 0.1 *M* $HClO_4$ and 50% methanol taken at a 3-mm diameter glassy carbon electrode at 100 mV s^{-1}, for a freshly abraded electrode, and following 12 min of scanning to 700 mV.

echin was easier to oxidize than caffeic acid [$(E_{p,a} + E_{p/2})/2$ was 35 mV less positive at 511 mV], whereas the epicatechin system was less reversible showing a ΔE_p of 55 mV. However, after several repeat scans over a period of 12 min, with an upper limit of just 700 mV, more energy (a more positive electrode potential) was required to oxidize epicatechin and the peak separation increased to 234 mV, pointing to high irreversibility brought about by inactivation of the carbon electrode; E_{mid} now provides a superior estimation of the formal potential. After abrading the electrode on alumina powder, the former response was recovered, pointing to the need to clean the electrode carefully prior to each cyclic voltammogram. A similar peak broadening was seen with catechin, and with the other antioxidants to a lesser extent. The ease with which the carbon electrode can be contaminated suggests that caution must be exercised in interpreting results when using this electrode material in ECDs for HPLC.

The effect of varying the scan rate in the cyclic voltammetry of antioxidants can be seen for 0.5 m*M* quercetin (Fig. 5). For a reversible system under diffusion control, the expectation is that $I_{p,a}$ will increase with the square root of the scan rate ($v^{1/2}$), which was indeed the case with quercetin (see the inset in Fig. 5). $E_{p,a}$ is expected to be independent of the scan rate, but a slight increase in the peak potential at faster scan rates was seen with quercetin. Further, if we focus on the curve obtained at 100 mV s^{-1}, we can see that quercetin was a little easier to oxidize than epicatechin [$(E_{p,a} + E_{p/2})/2$ was just 6 mV less positive at 505 mV]. However, the return cathodic peak was now much smaller than that for caffeic acid, rutin, or epicatechin, an effect that was more pronounced at the slower scan rates. It has been suggested that this loss of reversibility at slower scan rates is due to a chemical rearrangement following oxidation of quercetin (18), which will produce a species that is no longer reducible at the carbon electrode. Two further peaks were seen with quercetin when the potential was scanned to higher values, which involve first the oxidation of the hydroxyl group on the C ring (this peak is absent in the case of rutin where this oxygen group is linked to a rutinose disaccharide; see Chart 1), and then oxidation of the *meta*-diphenol on the A-ring (18).

The instability of the oxidation product was more marked in the case of the triphenol gallic acid (Fig. 6A). Gallic acid showed an anodic peak very similar in size and shape to that produced by caffeic acid, including a $|E_{p,a} - E_{p/2}|$ value of 36 mV, indicative of rapid electron

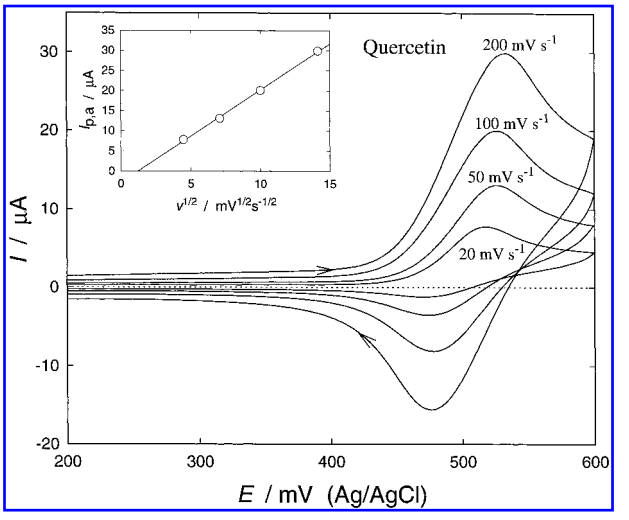


FIG. 5. Cyclic voltammogram of 0.5 m*M* quercetin in 50% 0.1 *M* $HClO_4$ and 50% methanol taken at a 3-mm diameter glassy carbon electrode and at scan rates from 20 to 200 mV s^{-1}. The **inset** gives the anodic peak current as a function of the square root of the scan rate ($v^{1/2}$).

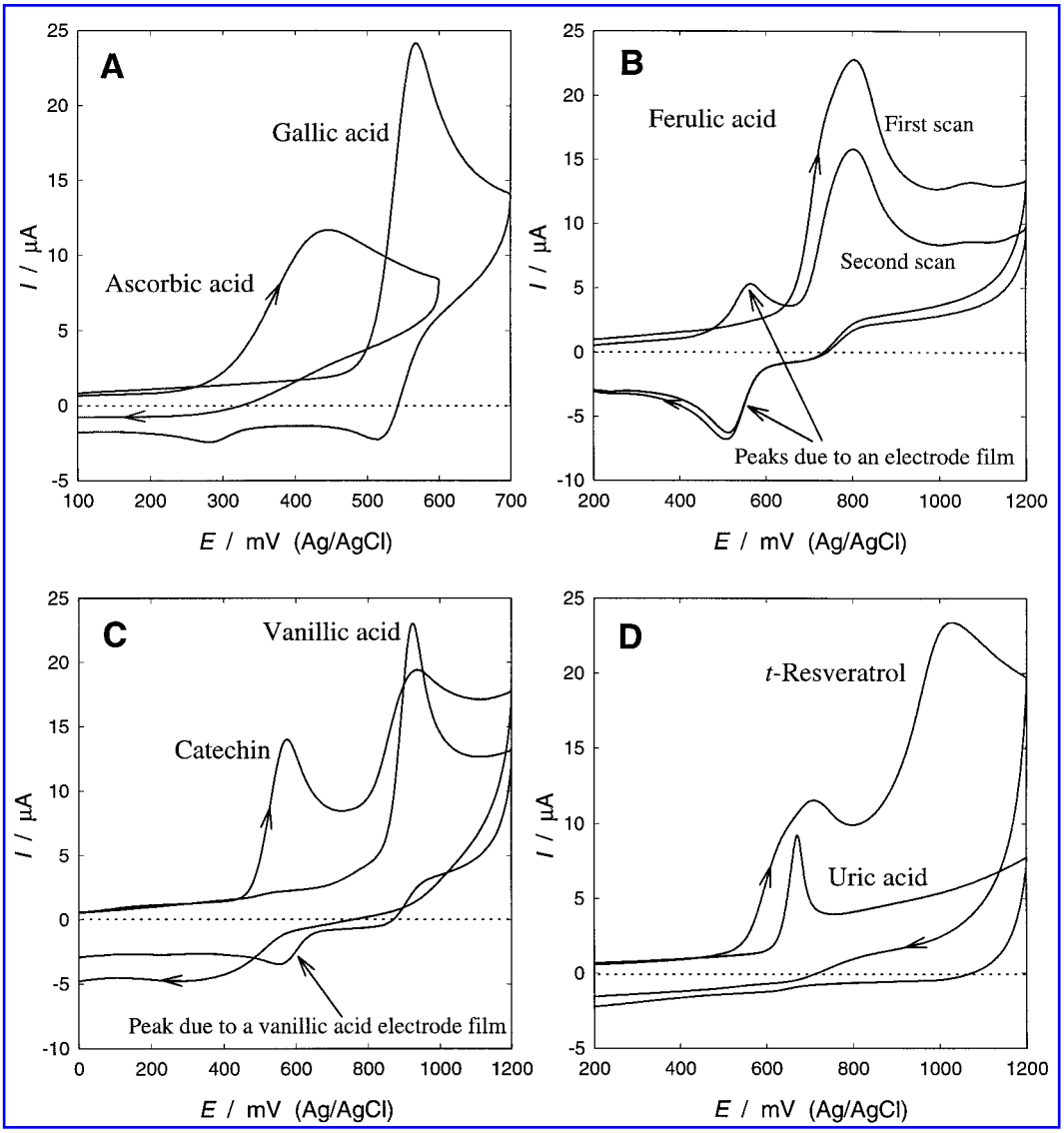


FIG. 6. Cyclic voltammograms of 0.5 m*M* solutions of antioxidants in 50% 0.1 *M* $HClO_4$ and 50% methanol taken at a 3-mm diameter glassy carbon electrode at 100 mV s^{-1}. (**A**) Gallic acid and ascorbic acid, (**B**) Ferulic acid, with a second scan taken straight after the first scan, (**C**) Catechin and vanillic acid, (**D**) *trans*-Resveratrol and uric acid.

transfer for the oxidation process. However, the return cathodic peak was significantly smaller, suggesting that the oxidation product was being rapidly degraded to another chemical form, which may only be reducible at much more negative potentials (possibly associated with the small cathodic peak seen at potentials less than 300 mV). Whereas gallic acid is oxidized at potentials similar to that of many *ortho*-diphenols, a triphenol group on the B-ring of the flavonoids produces even lower oxidation potentials, as shown by the lower oxidation potential of myricetin over quercetin (19), and by hydrodynamic voltammograms of green tea phenolics such as epigallocatechin gallate and epigallocatechin, which oxidize at lower potentials than epicatechin (28). We have also noted that extracts from the red leaves of native New Zealand plants containing high levels of the anthocyanin delphinidin (with a triphenol on the B-ring) also show an oxidation peak at potentials lower than for current due to phenolics containing *ortho*-diphenols.

A different type of irreversibility was seen with ascorbic acid (Fig. 6A). In this case, the oxidation process started much earlier than for

the other antioxidants tested [$(E_{p,a} + E_{p/2})/2$ at 401 mV was 104 mV less positive than even quercetin]. However, this ease of oxidation was not matched by reversible electrode kinetics, and a very broad anodic peak was produced ($|E_{p,a} - E_{p/2}| = 90$ mV). Although the oxidation product of ascorbic acid, dehydroascorbic acid, is known to be relatively stable, with hydrolysis to 2,3-diketogulonic acid and other products occurring over a period of hours at acidic pH, the complete lack of a cathodic peak indicates that this compound is largely electroinactive, at least in the potential range down to −100 mV. Specific chemical reductants such as cystine or mercaptoethanol are known to reduce dehydroascorbic acid to ascorbic acid, whereas in nature the enzyme dehydroascorbic acid reductase makes use of glutathione as a source of reducing power. The above estimate of 401 mV for the formal potential for ascorbic acid can be compared with a value of 317 mV (Ag/AgCl) at pH 1.4, derived from the literature using $pKa_1 = 4.04$ and the value of 282 mV on the hydrogen scale often quoted for oxidation of the ascorbate monoanion at pH 7 (7, 15), a value derived by Williams and Yandell (44) from earlier experimental data of Ball (2). The more positive value obtained with cyclic voltammetry in this case can be explained by the rather broad peak seen for ascorbic acid at this concentration.

The very low formal potential for ascorbic acid is consistent with its instability in foods and other aqueous media, where exposure to heat and light is also known to increase its rate of loss. Ascorbic acid can also reduce the oxidized quinone forms of many of the antioxidants considered in this article, effectively regenerating the antioxidant. Ascorbic acid is thought to play this role in biological systems by reducing oxidized tocopherols at the water–lipid interface, and to inhibit enzymatic browning in foods by reduction of *ortho*-quinone products. However, ascorbic acid may react directly with molecular O_2 and can also reduce Fe^{3+} to Fe^{2+}, which in turn can react with O_2 to form superoxide, H_2O_2, and the highly reactive $OH^{\bullet}$ (34), meaning that ascorbic acid can display prooxidant activity under certain circumstances. When ascorbic acid is added to white wines to lessen browning from oxidation of phenolics, sulfur dioxide is also added to "mop up" any H_2O_2 produced, or else the ascorbic acid can have a prooxidative effect and make browning worse.

A number of the antioxidants showed an anodic peak when the potential was scanned as far as 700 mV, whereas others did not (Table 1). Phenolics that have two hydroxyl groups on the *ortho* position of a benzene ring (such as the B-ring of catechin and quercetin on Chart 1) are known to have formal potentials in the 500–600 mV range in this solution (12). When the potential was scanned to 1,200 mV or more, further anodic peaks were seen due to the oxidation of *meta*-diphenol or isolated hydroxyl groups, often in a one-electron process. Ascorbic acid and caffeic acid did not show a second peak (Table 1). Other antioxidants displayed a first anodic peak only at higher potentials, showing that they were more difficult to oxidize and may be less reactive as antioxidants. A further consequence of scanning to higher potentials was increased contamination of the electrode surface by oxidation products, which lowered the currents seen on subsequent scans to a greater or lesser extent. This lowering of electrode activity makes it difficult to obtain consistent results when high positive potentials are used, which has implications in the use of ECDs for HPLC.

In the case of ferulic acid (Fig. 6B), a single, relatively broad peak ($|E_{p,a} - E_{p/2}| = 90$ mV) was seen with $(E_{p,a} + E_{p/2})/2$ at 740 mV. The cathodic peak seen on the reverse scan was matched by a new anodic peak that appeared on second scan in the forward direction. This set of peaks is likely due to oxidation products of ferulic acid deposited on the carbon electrode as a thin film. Peaks with an oxidation potential lower than that of the starting material are commonly seen with organic polymers such as polyaniline or polypyrrole, formed by oxidation of the respective monomers. Although these films are highly conducting and can be grown to sizeable thicknesses, the small peaks seen with ferulic acid may be due to oligomeric forms adsorbed on the electrode surface, which are easier to oxidize than ferulic acid itself. The ferulic acid film material is not expected to promote further film growth, and the size of these peaks remained unchanged

upon continued cycling. The anodic peak due to ferulic acid itself was also less intense on the second scan, consistent with a less active electrode surface.

Vanillic acid, like ferulic acid, has only one hydroxyl group adjacent to a methoxy group on a single benzene ring (Chart 1), but has less overall conjugation in the molecule. The oxidation peak for vanillic acid, while sharp with $|E_{p,a} - E_{p/2}| = 44$ mV pointing to rapid electron transfer, occurred at a value of $(E_{p,a} + E_{p/2})/2 = 901$ mV, at a similar position to *p*-coumaric acid, and in the region of the second peak for several phenolics such as catechin (Fig. 6C). An anodic peak at this position has been associated with hydoxyl groups in the *meta* position (as on the A-ring of catechin), or with isolated hydroxyl groups. We can also see on the reverse scan with catechin that the cathodic peak expected at ~460 mV is poorly defined, pointing to contamination of the electrode at the more positive potentials.

Cyclic voltammograms of two further antioxidants are shown in Fig. 6D. The first is *trans*-resveratrol, a molecule that has been the focus of considerable attention in the 1990s owing to its role as a phytoalexin in grapes and its presence in several herbal medicines (39). With $(E_{p,a} + E_{p/2})/2 = 653$ mV, *trans*-resveratrol was a less powerful reductant compared with molecules with *ortho*-diphenol groups and formal potentials less than 600 mV. The first peak was very broad and irreversible ($|E_{p,a} - E_{p/2}| = 114$ mV), with features similar to those seen in hydrodynamic voltammograms (45). Uric acid, on the other hand, displayed very fast kinetics given by the sharpest peak of those examined in this study ($|E_{p,a} - E_{p/2}| = 26$ mV), but also generated a product that was not reduced on the reverse scan. The large gap of >250 mV between the formal potentials of uric and ascorbic acids, major contributors to the antioxidant status of blood plasma, remains at physiological pH (20).

The results given in Table 1 also include two "artificial" antioxidants, BHT, often added to stabilize lipid-containing systems, and 3,5-dihydroxybenzoic acid (*meta* groups on a benzene ring and no further conjugation), which showed the highest oxidation potential. It was also found that when the pH of the solution was changed (by adding $NaClO_4$ in place of $HClO_4$), the potentials shifted by 54–58 mV per pH unit for most of the antioxidants tested, as expected for Eq. 3.

The values obtained above are consistent with previous reports of the oxidation of phenolics at carbon electrodes designed to describe the response seen in ECDs for HPLC (12, 29, 38). Besides polyphenols (16, 18, 23, 25, 37, 46), cyclic voltammetry has been applied to lipid-soluble antioxidants (26), vitamin C determination (35), and crude drugs (32), to show their effectiveness as antioxidants by the position of their oxidation peaks. Phenolics with low oxidation potentials have been shown to be more effective antioxidants by other measures of antioxidant status, such as the inhibition of lipid peroxidation (5), and reduction of ferrylmyoglobin (23). As a rapidly obtained measure of the tendency of an antioxidant to act as a reducing agent, cyclic voltammetry is analogous to Fe^{3+} reducing ability given by the FRAP (ferric reducing ability of plasma) assay, which also does not respond well to many sulfur-containing antioxidants. It likewise differs from assays that involve an oxidizable substrate and inhibition of a prooxidant, such as the TRAP (total radical trapping parameter) and ORAC (oxygen radical absorbance capacity) assays (36); these provide more direct measures of biological antioxidant activity but are more lengthy procedures.

A comparison of the above sort can be made between the formal potentials obtained in this study and published results for the total antioxidant status of antioxidant standards given using the commercial RANDOX kit (40). In this procedure, metmyoglobin reacts with H_2O_2 to form a radical that in turn reacts with a chromogen to form the colored $ABTS^{\bullet+}$ radical [ABTS = 2,2′-azinodi(3-ethylbenzthiazoline sulfonate)], a process that is suppressed by the addition of antioxidants. As seen in Table 2, phenolics with a lower formal potential showed a higher antioxidant status. The exception was ascorbic acid, which, despite the lowest formal potential, had the lowest antioxidant status using this test. In this case, the ability of ascorbic acid to reduce the Fe(III) of metmyoglobin in the RANDOX kit may lead to the additional production of free radicals and a low

Table 2. Comparison of Formal Potentials and Total Antioxidant Status Values

	E°′ *in mV (Ag/AgCl)*	*TAS (mmol L^{-1})*
Ascorbc acid	401	0.76
Quercetin	505	4.24
Epicatechin	511	4.96
Catechin	539	3.50
Caffeic acid	546	3.64
Gallic acid	550	3.00
trans-Resveratrol	653	2.54
Ferulic acid	740	1.84
Vanillic acid	901	0.92
p-Coumaric acid	910	1.56

The formal potentials [$E^{\circ\prime}$ estimated by $(E_{p,a} + E_{p/2})/2$] obtained in the present study are compared with the total antioxidant status (TAS) values given by Soleas *et al.* (40) using the commercial RANDOX kit and calibrated against TROLOX.

result. Although the RANDOX kit operates at neutral pH, a similar ranking is expected for cyclic voltammetry at this pH. Differences may occur where there is a change in protonation, dependent upon the respective p*K*a values, which will produce a different rate of change of oxidation potential with pH. For example, if Eq. 1 is changed to a 1 proton/2 electron process (as for ascorbic acid beyond pH 4), a decrease of 29 mV per pH unit is expected in the formal potential, rather than 59 mV per pH unit as for a 2 proton/2 electron process at lower pH. As the pH is changed, the phenolics may also oxidize by different reaction pathways. It is thus preferable to measure oxidation potentials in the particular solvent of interest rather than to extrapolate results obtained under other conditions too far.

A further application of cyclic voltammetry has been proposed to describe the antioxidant properties of complex biological samples, such as blood plasma and saliva (10, 26, 27), and edible plant extracts (11). A decrease in the reducing power of various organs with aging and in diabetic plasma, along with changes in the rat brain following injury, have all been observed (20). These have provided information important to assess the redox status of the cell under various stresses, and to test the free radical theory of aging.

When cyclic voltammograms were taken of white and diluted red wines, an oxidative current was seen from 300 mV, due principally to the oxidation of phenolic antioxidants as each formal potential was approached (Fig. 7A). Owing to the mixture of compounds that contribute to the response, a series of waves rather than distinct peaks were seen. The current at 400–500 mV is a measure of the level of the more powerful reductants (phenolics with *ortho*-diphenol groups, with formal potentials ~130 mV less positive than those listed in Table 1 due to the shift to a pH 3.6 solution). Different antioxidants at the same concentration will also contribute a varying current response dependent upon their diffusion coefficients (as given by Eq. 4), and as seen for the variation in the peak current ($I_{p,a}$) for the various antioxidants reported in Table 1. Additional activity in Fig. 7A at ~650 mV in the red wine can be associated with malvidin anthocyanins, and at

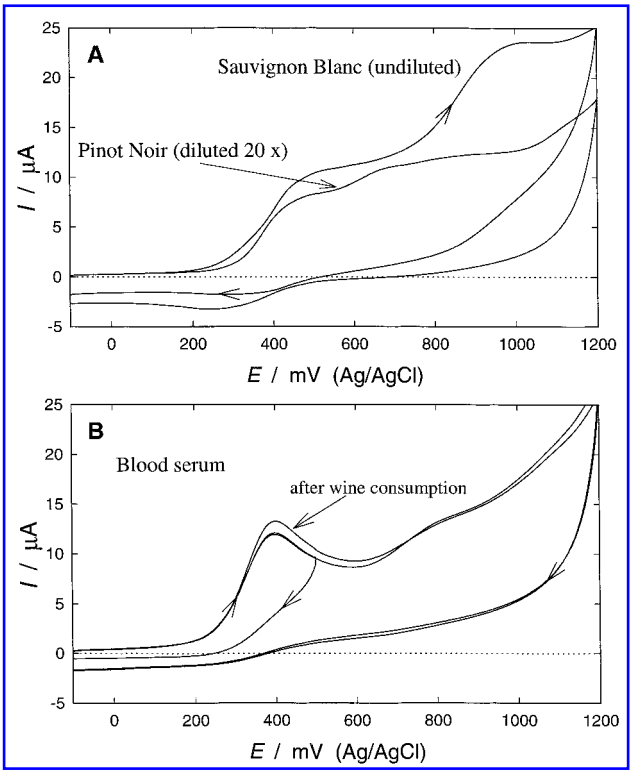


FIG. 7. Cyclic voltammograms taken at a 3-mm diameter glassy carbon electrode at 100 mV s^{-1} of: (**A**) a 1998 Pinot Noir red wine diluted 20 times in a model wine solution (12 % ethanol, 0.05 *M* tartaric acid, with added NaOH to give a pH of 3.6), and an undiluted 1998 Sauvignon Blanc white wine; and (**B**) blood serum—one of the scans was reversed at 500 mV to show the lack of a cathodic peak on the reverse scan.

800–1,000 mV with antioxidants of higher formal potentials. To adequately quantify various classes of phenolics found in wines, it is necessary to dilute the wine even further (25).

A cyclic voltammogram of blood serum is shown in Fig. 7B. The initial peak, here at 400 mV, has been ascribed to ascorbic and uric acids (10), and is a measure of the antioxidant "health" of the blood. This peak was relatively broad and showed no cathodic current on the reverse scan when the potential was stopped at 500 mV (ascorbic and uric acids on their own also show no cathodic peak). Again antioxidants with higher formal potentials contributed to the current seen beyond 700 mV on the wider scan. Initial results show that after the ingestion of two glasses of red wine, the current at the first peak increases by 10%, indicating that the antioxidant capacity of the blood has been enhanced, consistent with previous reports using alternative measures of antioxidant capacity (9, 42, 43).

Perspectives

At the level of monitoring antioxidants and neurochemicals *in vivo*, cyclic voltammetry at carbon microelectrodes (5–50 μm) in extracellular fluid is well established (33). As the potential is scanned, peaks are seen due to ascorbic acid (present in high concentrations), uric acid, and then catecholamines such as dopamine and noradrenaline (which also contain oxidizable *ortho*-diphenol groups), and a range of metabolites. To improve selectivity, the microelectrodes can be modified with Nafion, which excludes the ascorbate and urate anions, but allows the cationic forms of various neurotransmitters to reach the electrode. As further electrode modifications are developed to create more selective electrodes, we can expect more antioxidants involved in cellular signaling pathways to be quantified *in vivo* by using this technique.

In conclusion, the application of electrochemistry is providing an important means of characterizing many antioxidants. The ability of antioxidants to be oxidized at inert electrodes allows them to be monitored using ECDs, following HPLC separation, and to be characterized using cyclic voltammetry. Where reducing power is a key aspect of the effectiveness of an antioxidant in biological systems, this can readily be determined at a carbon electrode. Cyclic voltammetry of complex mixtures, including food samples, blood plasma or serum, and extracellular fluid, can be used to rapidly estimate the antioxidant status due to compounds of a certain reducing power. This is directly applicable to monitoring the redox status of cells, particularly in conjunction with other measures for enzymatic and metal-chelating antioxidants.

ACKNOWLEDGMENTS

The assistance of Dr. Anthony Phillips, of the School of Biological Sciences, The University of Auckland, who took blood samples, and of Honglei Zou, who provided cyclic voltammograms of the wine samples, is gratefully acknowledged.

ABBREVIATIONS

A, electrode area; BHT, butylated hydroxytoluene; C_R*, bulk concentration of antioxidant (reductant); D_o, diffusion coefficient; E, potential; E^o, standard potential; $E^{o\prime}$, formal potential; $E_{1/2}$, half-wave potential; E_{mid}, midpoint potential; $E_{p/2}$, half-peak potential; $E_{p,a}$, anodic peak potential; $E_{p,c}$, cathodic peak potential; ΔE_p, peak potential separation; ECD, electrochemical detector; F, Faraday constant; HPLC, high performance liquid chromatography; I, current; $I_{p,a}$, anodic peak current; $I_{p,c}$, cathodic peak current; n, electrons passed; O, oxidant; R, reductant; R, gas constant; T, absolute temperature; v, scan rate; ω, rotation rate.

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Received for publication March 1, 2001; accepted May 15, 2001.

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