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Square-Wave and Cyclic Voltammetry of Native Proanthocyanidins Extracted from Grapevine (Vitis vinifera) on the Glassy Carbon Electrode

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Abstract: Condensed tannins are short polymers of flavan-3-ols found in grapes (also known as proanthocyanidins). An investigation on the electrochemical oxidation of grapevine proanthocyanidins (PAs) on glassy carbon electrodes under various conditions was conducted for the first time. To study how the proanthocyanidins were oxidized, square-wave and cyclic voltammetry were used. There is a predominant oxidation peak associated with the extract of proanthocyanidins, and this can be attributed to the oxidation of catechol 3′,4′-dihydroxy groups, which can form their oxidation peak. There are two electrons and two protons involved in the oxidation of the catechol group, which must be kept in mind when considering the oxidation of the catechol group. On the glassy carbon electrode (GCE), the PAs extracted from grapevine are oxidized by an adsorption-dependent mechanism as they interact with the GCE surface. As a result, it was found that the anodic peak current varied linearly with PAs’ concentrations in the range of 4 to 50 ppm, with a detection limit of 3.07 ppm (S/N = 3). There was a development in the surface concentration of the oxidation products at the GC electrode; as the scans progressed, the surface concentration of oxidation products at the electrode remained at 4.83 × 10⁻¹¹ mol cm⁻², indicating that they were immobilized on the GCE as oxidation products adsorbed on the electrode.

Keywords: voltammetry; condensed tannins; oxidation mechanism; flavonoids; Vitis vinifera

1. Introduction

Proanthocyanidins (PAs) are polyphenolic compounds with a complex structural profile widely found in plants [1]. Tannins are classified into two categories: hydrolyzable tannins and condensed tannins [2]. As the first group of tannins, hydrolyzable tannins (HTs) are polyesters derived from gallic and ellagic acids, respectively (ellagitannins (ETs) and gallotannins (GTs)). As for the latter group, condensed tannins, also known as proanthocyanidins (PAs), are oligomers (2 to 5 units) and polymers (6 to 60 units) that contain flavan-3-ols as their basic skeleton. Typically, flavon-3-ol nuclei consist of C6–C3–C6 phenolic structures (Figure 1) [3]. In order to determine the structure of proanthocyanidins (PAs), several structural factors need to be considered. These factors include the connection pattern between units, the type of flavan-3-ol unit, the composition of the phenyl hydroxyl group (e.g., ester or methyl), and the spatial configuration. Proanthocyanidins (PAs) are generally connected between carbon atoms C4 (in the C-ring) of the preceding unit and carbon atoms C6 or C8 of the following flavan A-ring [4]. Stereochemically, the C4–C8 links are preferred, but they are not exclusive. It is common for both linkages (C4–C8 and C4–C6) to exist in a 3:1 ratio [2].
Recently, PAs have received considerable attention due to their anti-inflammatory [5,6], anti-infectious [7], anti-carcinogenic [8,9], and cardioprotective properties [10,11]. It is believed that these compounds are protective as a result of their ability to scavenge free radicals and inhibit lipid peroxidation [12–15]. Several epidemiological studies have demonstrated that fruits, vegetables, and certain beverages can protect against chronic diseases, primarily due to their antioxidant contents, such as polyphenols [16]. In the American diet, polyphenols are one of the most significant sources of antioxidants [17]. PAs are one of the most common sources of polyphenols due to their widespread distribution [18]. The biological properties of PAs, including their healing abilities, are attributed to their ability to form complexes with metal ions and macromolecules such as proteins [19–21]. Therefore, tannins are widely used in tanning leather, medicine, and the food industry. Many natural drugs utilize tannin extracts for the treatment of diarrhea [22], diuretics [23,24], stomach pain and tumors [25], and antiseptic properties [26]. As tannins can precipitate heavy metals and alkaloids (except morphine), they can also be used for intoxication [27]. Both antioxidative activity and electrochemical oxidation of polyphenols involve the breakdown of O-H bonds [28,29].

PAs or condensed tannins from plants or food samples must be characterized and quantified for three purposes. The first application of PAs is in taxonomic studies or studies of structure-function relationships [30], including environmental stress responses [31,32]. Secondly, we aim to investigate the potential health benefits of plant extracts and tannin-rich formulations for commercial products such as cosmetics and nutritional supplements [33–35]. Third, it is crucial to develop specialty chemicals or polymeric materials that are produced from renewable phenolic compounds in accordance with each tannin’s characteristics and properties [36–38].

Figure 1. Chemical Structures of (A) C6–C3–C6 phenolic ring of flavonoids, (B) Polymeric PAs connected through C4→C8 linkage.
In order to separate, identify, and quantify polyphenolic compounds, different techniques have been used, but high-performance liquid chromatography (HPLC) is the most commonly used [39]. Additionally, the determination of antioxidant activity has been carried out in vitro by many methods, such as spectrophotometry [40] and electroanalytical techniques such as cyclic voltammetry (CV). Compared to traditional spectrophotometric methods, CV has proven to be a successful method of measuring antioxidant capacity in plant extracts, wine, and juices [41–47]. In order to reveal the antioxidant properties of an antioxidant, electrochemical measurements can provide some essential physicochemical parameters (redox potentials, electron-transfer rate constants, electron numbers, etc.) for the investigation [48]. Anodic peak current intensities and peak oxidation potentials can be evaluated by voltammetry to obtain information about antioxidant capacities [49–51]. Furthermore, if the peak potential and current values of the antioxidants are calculated, we can use these values as an indication of the contents of antioxidants. As a consequence, voltammetry can effectively be used to determine both the antioxidant capacities of different substances as well as to determine the antioxidant content of the substances, so it is a convenient and helpful method. These accomplishments led us to the development of voltammetry as a tool for addressing the redox properties of grapevines and for determining the amount of PAs present in grapevines using voltammetry as a tool. This study examines the electrochemical oxidation of PAs since previous studies have not examined them. Under various experimental conditions (such as pH, scan rate, and concentration), square wave and cyclic voltammetry measurements have been conducted to elucidate the electrochemical oxidation mechanism of proanthocyanins. We also developed a simple, low-cost, but competitive detector based on a glassy carbon electrode (GCE) that does not require any modification in order to measure condensed tannins without the need for a model compound in order to quantify condensed tannins in plant extracts.

2. Materials and Methods

2.1. Chemicals and Solutions

Without any further purification, all chemicals and reagents were used as received from Sigma-Aldrich (St. Louis, MO, USA). In addition, Triton™ X-100, ascorbic acid with a purity greater than 99%, EDTA with a purity of ≤100%, chloroform with a purity of 99.9%, hexane with a purity of 99.5%, acetone with a purity of 99.9%, and methanol with a purity of 99.9% are among the products supplied. The following buffer solutions were used in this study: the Britton–Robinson buffer (B-R) contains 40 mmol/L of H$_3$BO$_3$, CH$_3$COOH, and H$_3$PO$_4$, and the phosphate buffer contains 0.3 mol/L of H$_3$PO$_4$/KH$_2$PO$_4$·H$_2$O. By adding 0.2 mol/L of HCl or NaOH to the solution, the pH was adjusted. A fridge at 5°C was used to store all solutions.

2.2. Instruments

The electrochemical experiments were conducted using an Autolab PGSTAT128N Electrochemical Workstation powered by Nova 2.0 software (Eco-Chemie, Utrecht, The Netherlands). In this study, we utilized a three-electrode configuration cell consisting of a glassy carbon electrode (GCE, 3 mm diameter) as a working electrode, a Ag/AgCl electrode as a reference electrode, and a platinum electrode as a counter electrode. Before each run, the glassy carbon electrode was manually polished two times with aqueous suspensions of 1.0 µm alumina and 0.05 µm diamond over two minutes each. In the following steps, the GCE was rinsed in ultrapure water and sonicated for five minutes in ethanol and ultrapure water. Cyclic voltammetry was used for electrochemically cleaning the GCE B-R buffer (pH 1.80) for 25 cycles at a scanning rate of 50 mV s$^{-1}$, and the potential range was –1 to 1 volts. A Britton–Robinson buffer (B-R) solution was used for the cyclic voltammetry (CV) and square-wave voltammetry (SWV) experiments. Electrochemical measurements were conducted after degassing the solutions with high-purity nitrogen. Subsequently, nitrogen was maintained as a blanket. The temperatures at which all experiments were conducted were room temperature. In order to measure the pH of solutions, the pH meter HI 2221,
manufactured by Hanna Instruments (Bucharest, Romania), was used. An ultrapure water system (Biopak Polisher Millipore, Billerica, MA, USA) was used to prepare all aqueous solutions with a resistivity of 18.2 MΩ cm at 25 °C.

2.3. Plant Material

Ripe fruits from *Vitis vinifera* L. were handpicked from a local vineyard in Alexandria Governorate, Egypt. After immersing the fruits in liquid nitrogen, the seeds were gently broken off while still frozen. An extraction of PAs was carried out by immersing 200 g of Pericarp bits in liquid nitrogen and milling them finely in a mortar. The resulting pericarp powder was stored at -20 °C, ready to be extracted.

2.4. Extraction and Purification of Proanthocyanidins (PAs)

Extraction and purification of proanthocyanidins (PAs) were performed as described in Ref. [52]. Briefly, an aliquot of powdered pericarp (100 g) was added to a cold buffer (pH 7.84) containing 0.3 M potassium phosphate buffer, 1% Triton X-100, 1.5% ascorbic acid, and 10 mM EDTA. Using vigorous shaking, we homogenized the resulting mixture, removing any frozen materials and filtering the mixture. To remove the green liquid from the cake, vacuum suction was used, and 100 mL of water, 100 mL of chloroform, and 50 mL of hexane were used to wash the cake three times. Following the addition of the cake to 150 mL of acetone/water mixture (7:3), the slurry was gently swirled in the crucible for an hour. Following vacuum suction, 150 mL of acetone/water was used to wash the leftover cake (primarily cell walls). The acetic acid extract was vacuum concentrated before being frozen. After lyophilized samples were dissolved in hot 100 mL methanol, 300 mL of chloroform was added, and the filtrate was filtered off. For washing the PA precipitate, 100 mL chloroform/methanol (3:1) and 50 mL ether were used. For further research, the purified PAs were stored under argon at 20 °C after being dried in a hood. The PAs solutions used in the current experiments were prepared freshly according to the buffer solution volume in the electrochemical cell, with a concentration of (ppm, mg/L).

3. Results and Discussions

Flavonoids containing ortho-dihydroxyl groups show a strong correlation between their structure and their oxidation ability. The oxidation mechanism has been demonstrated by several published studies to involve two electrons being transferred stepwise through a one-electron process leading to the oxidation of the catechol moiety, followed by an irreversible chemical reaction resulting in the formation of the o-quinone. As shown in Scheme 1, catechols are oxidized in order to produce their corresponding o-quinones via the oxidative reaction. This means that extracts of proanthocyanidins (PAs), which contain a similar catechol moiety, will oxidize at the glassy carbon electrode (GCE).

![Scheme 1](image)

*Scheme 1.* The oxidation mechanism of catechol moiety.

3.1. Voltammetric Behavior of Native Proanthocyanidins

A study on the electrochemical behavior of PAs extract was conducted at GEC using SWV in the B-R buffer solution. The SWV technique has several advantages, including the fact that it consumes less electroactive species, is faster, and has fewer problems associated with electrode poisoning than other methods [53]. Additionally, due to the fact that the current can be sampled both positively and negatively, oxidation and reduction peaks of the electroactive species can also be obtained in the same experiment, and electron reversibility can be evaluated with only one scan of the electrode. The PAs extract oxidized during the SWV measurements due to the positive sweep of the GCE potential. In B-R buffer
result of continuous scanning, the current can be sampled both positively and negatively, oxidation and reduction peaks of PAs were observed at 0.52 V since PAs have free OH groups that can adsorb on GCE surfaces. As a result of continuous scanning, the peak corresponding to the oxidation of PAs appeared at 0.52 V.

Moreover, the peak ratio ($i_{pc}/i_{pa}$) was below unity. According to these results, PAs undergo quasi-reversible redox reactions on GCEs. For the oxidation wave, a linear relationship on the logarithm of the forward current ($\log i_{f} (A)$) versus the logarithm of scan rate ($\log \nu (V s^{-1})$) was observed when plotting the logarithm of the anodic peak current ($\log i_{p}$ (A)) versus the logarithm of scan rate ($\log \nu (V s^{-1})$) (Inset of Figure 2B). As a result of adsorption-controlled electrodes, this value is very close to the theoretical value of 1.0. In accordance with these findings, PAs extracted from the grapevine are oxidized on GCE by an adsorption-controlled mechanism.

### 3.2. Repetitive Cyclic Voltammograms of Proanthocyanidins

A CV experiment on GCE with 40 ppm PAs was conducted for 20 cycles at a scan rate of 20 mV s$^{-1}$ in a B-R buffer (pH 1.8) in the potential range of 0.2 to 0.8 V (Figure 3A). A peak corresponding to the oxidation of PAs appeared at 0.52 V since PAs have free OH groups that can adsorb on GCE surfaces. As a result of continuous scanning, the current of this peak increased. Afterward, the PAs were electropolymerized efficiently on the electrode surface [55], explaining why the PAs are not able to be accessed further due to the polymer layer that covers the electrode surface. The fabricated polymer of
PAs on the glassy carbon electrode (PAs/GCE) was washed with water and transferred into a blank B-R buffer (pH 1.8), and reversible peaks were observed at 0.52 and 0.48 V (Figure 3B). After continuously scanning five cycles in a potential range of 0.2 to 0.8 V in the blank buffer, this pair of peaks gradually reached stability with a 10% decrease in order to increase reproducibility. In both the dry and B-R buffer states, the modified PAs/GC electrode displayed high stability. For the continuous cyclic sweeps, there was no loss of electrode electroactivity; the same peak can be seen at PAs/GCE, which indicates that an electro-active polymer film exists there. According to Sharp et al. [56], the electrode surface coverage was estimated. Using Equation (1), the peak current can be correlated with the concentration of electroactive species on the electrode surface (i.e., surface coverage, \( \Gamma \)):

\[
i_{p}^{a} = n^{2}F^{2}A\Gamma \nu / 4RT
\]

where \( n \) is the number of electrons involved in the reaction, \( A \) is the electrode surface area in cm\(^2\), \( \Gamma \) is the electrode surface coverage in mol cm\(^{-2}\), and other symbols have their usual meanings. Using cyclic voltammetry (CV) in B-R buffer (pH 1.8) at different scan rates, an electrochemical study of PAs/GC modified electrodes was conducted (Figure 3C). There is a linear relationship between the anodic peak current \( (i_{p}^{a}) \) of the adsorbed PAs and scan rate \( (\nu) \), \( i_{p}^{a} (A) = 1.78 \times 10^{-8} + 1.29 \times 10^{-5} \nu \) (V s\(^{-1}\)) with a correlation coefficient of 0.999 (Figure 3D). According to the slope of the anodic peak current against the scan rate, it is \( 1.29 \times 10^{-5} \). In this case, since there were only two electrons involved in the reaction, the calculated surface concentration of PAs was \( 4.83 \times 10^{-11} \) mol cm\(^{-2}\), further proving that they were immobilized.

Figure 3. (A) CVs of 40 ppm PAs on GCE in B-R buffer (pH 1.80) at scan rate 20 mV s\(^{-1}\) for 20 cycles. (B) Cyclic voltammetry of PAs/GCE in blank B-R buffer a (pH 1.8) at a scan rate of 20 mV s\(^{-1}\) for 5 cycles. (C) CVs PAs/GCE in blank B-R buffer (pH 1.80) at different scan rates (10–100 mV s\(^{-1}\)). (D) The linear regression plot of the anodic peak current \( (i_{p}) \) vs. scan rate \( (\nu) \).
3.3. Effect of pH on the Cyclic Voltammetric Behavior of Proanthocyanidins

Different pH values of supporting electrolytes were used for the voltammetric oxidation of PAs. Figure 4A illustrates SW voltammograms obtained at different pH values between 1.8 and 8.8 for 20 ppm PAs in 0.04 M B-R buffer solution. At all pH values, anodic peaks of PAs oxidation were observed, and the peak position varied with pH, which provided valuable insight into the electrode process. According to Figure 4B, plotting the oxidation peak potential ($E_p$) against pH shows a linear shift to a less positive value until pH 8.8. It confirms that deprotonation plays a crucial role in PA oxidation. A linear regression equation $E_p = 0.57 - 0.056 \text{pH}$ was derived from the relationship between $E_p$ vs. pH. As a result, PAs oxidize electrochemically to produce the corresponding o-quinone using a two-electron two-proton reaction. Additionally, the peak current decreases with increasing pH, indicating that pH has a major impact on the ionization of PAs' hydroxyl (OH) groups. PAs become more hydrophilic in an acidic medium due to their nonionizing OH groups, which migrate on the hydrophobic surface of a GCE and adsorb there. By becoming more ionizable in the basic medium, the OH groups make the PAs molecule more hydrophilic and migrate away from the surface of the GCE to the bulk solution [57]. As a result, the electroactive species of PAs (i.e., nonionizable forms) are more concentrated at acidic pH levels than at basic pH levels. Accordingly, pH 1.80 was chosen as the optimal value for voltammetric quantification of grapevine PAs.

![Figure 4.](image)

3.4. Effect of Proanthocyanidins Concentration and Limit of Detection

Quantification of PAs is complicated because of their structural complexity [58]. Consequently, only one published study used a simpler model molecule to quantify PAs [59]. As a model compound for quantifying PAs, catechin was used in this study to quantify PAs in commercial products of Acacia (*Acacia mearnsii* de wild). In order to prevent measuring PAs in a way that is equivalent to estimating the concentration of a model compound rather than determining the actual concentration of the PAs, PAs' quantification should be carried out without the use of any model compounds. Our study provides the first protocol for measuring PAs directly without using any model compounds since we extracted native PAs and purified them very well. Square-wave voltammetry (SWV), using the bare GCE, was used to produce the calibration curve for PAs based on the optimized conditions. According to Figure 5, square-wave voltammograms were obtained at various PA concentrations. As a result of the redox process associated with PAs, a well-defined peak can be observed for all voltammograms near +0.50 V. A linear calibration curve was observed from 4 to...
50 ppm PAs, with a correlation coefficient of 0.993 (Inset of Figure 5). According to the linear regression equation, the function can be expressed as follows:

\[ ip (A) = 1.06 \times 10^{-7} + 1.69 \times 10^{-7} [\text{PAs}] \text{ (ppm)}, r = 0.993 \] (2)

where \( ip \) is the resulting peak current in A, and \([\text{PAs}]\) is the proanthocyanidins concentration in ppm. Analytical curve parameters were used to calculate the limit of detection (LOD). \( \text{LOD} = 3 \frac{S_b}{s} \), where \( S_b \) is the standard deviation of the y-intercept and \( s \) is the slope. Based on the given conditions, the calculated LOD is 3.07 ppm.

A comparison is being made between the results of the current protocol with those from other protocols using catechin as a model compound. It is evident from the results of the current work that there are two main advantages, as can be seen in Table 1. As for the first advantage, the investigation does not use any model compounds in order to quantify PAs. For the second one, the glassy carbon electrode was used without any modification whatsoever, and it was found to be very effective. On the basis of the above results, a simple and precise electrochemical sensor for the detection of PAs in grapevines was developed on the basis of an unmodified glassy carbon electrode, and its capabilities were shown to be simple and precise.

Table 1. A comparison of this protocol with other protocols using catechin as a model compound.

| Electrode                                      | Model Compound | Linear Range (ppm) | Limit of Detection (ppm) | Reference |
|-----------------------------------------------|----------------|--------------------|--------------------------|-----------|
| Glassy carbon electrode modified with gold nanoparticles stabilized in carboxymethylcellulose | Catechin       | 0.09–2.9           | 0.08                     | [59]      |
| Glassy carbon electrode None                  | None           | 4.0–50.0           | 3.07                     | This work |
4. Conclusions

For the first time, we have characterized proanthocyanidins extracted from grapevine (*Vitis vinifera*) on the glassy carbon electrode using square-wave and cyclic voltammetry methods. In proanthocyanidins, the hydroxyl groups of the catechol moiety are predominantly responsible for the oxidation process, and two electrons and two protons are involved in an adsorption-controlled reaction. As PAs’ oxidation products adsorb on glassy carbon electrodes, they become immobilized on the electrode surface. According to the results, the GCE appears to be sensitive to PAs detection based on the results of the experiments. In this paper, we present the linear range and the detection limit based on bare GCE data. We are now able to better appreciate the antioxidant properties of proanthocyanins as a result of our understanding of their electrochemical properties. As a result, proanthocyanidins-containing food supplements derived from grape vines (*Vitis vinifera*) may provide health benefits by preventing free radicals from causing oxidative damage in the body.

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