Research Article

Yafen Fu, Zongyi You, Aiping Xiao, Liangliang Liu*, Weien Zhou*

Electrochemical evaluation of the antioxidant capacity of natural compounds on glassy carbon electrode modified with guanine-, polythionine-, and nitrogen-doped graphene

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Abstract: An electrochemical sensor based on guanine-, polythionine-, and nitrogen-doped graphene modified glassy carbon electrode (G/PTH/NG/GCE) was fabricated and applied for antioxidant capacity evaluation of natural compounds and complexes in electrochemical method since natural sources of active compounds exhibited various antioxidant activities. When the antioxidants existed in the system, the generated hydroxyl radicals were scavenged and the damage to guanine immobilized on the electrode was reduced less resulting in the oxidation peak current increased in square wave voltammetry. After the modifications of polythionine- and nitrogen-doped graphene, the oxidation peak current was improved. The effects of pH, incubation time, and concentrations of guanine and Fe^{2+} ions on the performances of the electrode were investigated and optimized. The G/PTH/NG/GCE showed good linearity, reproducibility, and storage stability for antioxidant capacity evaluation of ascorbic acid at the optimum conditions. The antioxidant capacities of three flavonoids and three plant extracts were measured using the G/PTH/NG/GCE and DPPH methods. Myricetin showed the highest antioxidant capacity in both electrochemical and DPPH methods. The proposed G/PTH/NG/GCE exhibited easy fabrication procedure, rapid detection time, and low cost for the detection of antioxidant activity for various kinds of samples.

Keywords: antioxidant capacity, electrochemical sensor, flavonoids, nitrogen-doped graphene, polythionine

*** Corresponding author: Liangliang Liu,** Characteristic Fruit and Vegetable Research Office, Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha, 410205, People's Republic of China, e-mail: liuliangliang@caas.cn, tel: +86-731-8899-8525
*** Corresponding author: Weien Zhou,** Hunan Fangsheng Pharmaceutical Co., Ltd, Changsha, 410205, People's Republic of China, e-mail: zwe197565@163.com, tel: +86-731-8899-7168
Yafen Fu, Zongyi You, Aiping Xiao: Characteristic Fruit and Vegetable Research Office, Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha, 410205, People's Republic of China

1 Introduction

Reactive oxygen species (ROS) were common in natural biological processes. However, when there was an accumulation of ROS induced by environment and chemicals, the oxidative stress would occur in the body and cause damage of proteins, lipids, and even cells [1]. This process could lead to various chronic diseases such as cancer, muscular dystrophy, heart disease, and atherosclerosis [2]. Antioxidant compounds existing in various fruits, vegetables, and beverages could react with ROS by scavenging free radicals, transition metal chelation, and singlet oxygen quenching [3]. Flavonoids and polyphenols compounds were the major class of antioxidants that came from diet possessing many activities in anti-inflammation and anti-virus [4]. Accordingly, efficient detection of antioxidant capacity for various samples could promote the discovery of new antioxidants and related researches. Different methods were proposed as tools for the analysis of antioxidant capacity for compounds or complex mixtures such as absorption spectroscopy and high-performance liquid chromatography [5]. However, the main problem of spectroscopic methods was the color of samples that would interfere with the reaction system and affect the precision of detection [6]. As an easy, sensitive, and fast technique, the electrochemical method could become a suitable option for evaluating the antioxidant content and the reducing ability of mixture [7]. Based on a specific redox reaction, there was no need for difficult sample preparation, and the disturbance in the detection was much less [8].

Graphene was a kind of carbon nanomaterial with high-specific surface area and conductivity, which has been used in electrochemical sensing [9]. Heteroatom doping and functionalization of graphene could make an improved performance in gravimetric capacitance and magnetic response by increasing conductivity, pseudocapacitive behavior, electron transfer, and so on [10]. Recently, nitrogen-doped graphene (NG) showed advantages in electrocatalysis owing to good chemical stability and high-electrical conductivity [11]. It has attracted particular research interests in electrocatalyst [12],

semiconductor [13], lithium-ion batteries [14], and other fields [15]. Therefore, it could be expected that NG could exhibit enhanced performance in chemical and electrical properties.

Thionine was a phenothiazine redox dye with good solubility in water and ethanol [16]. Polythionine (PTH) was widely applied in the modification of electrochemical sensors because of its good redox reversibility, fast charge transfer capacity, and good stability [17]. An electroactive and conductive PTH membrane could be easily accomplished on the surface of the electrode using electrochemical polymerization. This kind of fabrication showed advantages in the thickness of the film, response time, and charge transfer rate than the suspension coating method [18,19]. It has been reported that PTH-modified electrode was applied for the determination of endocrine-disrupting compounds, hydrogen peroxide, hepatitis B, and nicotinamide adenine dinucleotide [20–23].

In this study, NG and PTH membranes were consequently modified on glassy carbon electrode (PTH/NG/GCE) to combine the excellent properties of them in electrical conductivity and adsorption ability. Then, guanine was immobilized on PTH/NG/GCE (G/PTH/NG/GCE) and used as a stable G/PTH/NG/GCE electrochemical sensor in the evaluation of antioxidant capacity. During the detection procedures, the hydroxyl radicals could make damage guanine on electrode, resulting in the decrease of oxidation peak current in square wave voltammetry (SWV). When antioxidants existed in the reaction system, the number of hydroxyl radicals would decrease due to the antioxidant activity, and there would be less damages on guanine. As a result, a higher peak current in SWV was observed and the following calculation could be completed. After modification, the proposed G/PTH/NG/GCE showed higher oxidation peak current, which meant higher sensitivity for evaluation. It could be directly used in the determination of antioxidant capacity for compounds and mixture without extra processes and reagents. Therefore, this modified electrode could provide a fast and cheap sensor for the analysis of antioxidants with promising prospects in the food and chemical industries.

2 Experimental

2.1 Chemicals and apparatus

Ethylenediaminetetraacetic acid (EDTA), guanine, and thionine were purchased from Merck KGaA (Darmstadt, Germany). Ammonium hydroxide, hydrogen peroxide, sulfuric acid, potassium permanganate, urea, and nitric acid were purchased from Sinopharm Chemical Reagent (Shanghai, China). Graphite flakes were purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). Myricetin, kaempferol, and galangin were acquired from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Ultrapure water (18.25 MΩ cm) was obtained from a Millipore system (Millipore Corporation, Milford, MA, USA). All other chemicals were of analytical grade without further purification.

The electrochemical measurements were accomplished on a CHI 660E Electrochemical Workstation (Shanghai Chenhua Instrument Company, Shanghai, China) using a standard three-electrode system. The characterizations of materials were conducted by transmission electron microscope (TEM) and scanning electron microscope (SEM). The observations were completed by a JSM 6610Lv scanning electron microscopy (JEOL, Tokyo, Japan) operated at 25 kV and a JEM-2100 electron microscope (JEOL, Akishima, Japan).

2.2 Preparation of NG

Graphene oxide (GO) was prepared according to the Hummers method with some modifications [24]. About 2.0 g of graphite flakes and 8.0 g of $KMnO_4$ were added into 100 mL of ice-cold concentrated H_2SO_4. The mixture was kept in an ice bath and stirred for 3 h. Then, 400 mL of water containing 20 mL of 30% H_2O_2 was slowly added. The produced golden mixture was filtrated and the solid was washed with water and 5% HCl three times, respectively. The finally obtained solid was dried in vacuum at 60°C for 24 h.

For the preparation of NG, 0.1 g of GO was dispersed into 100 mL of water. About 2.0 g of urea was added into GO solution as the source of nitrogen and ultrasonically treated for 2 h. Then, the solution was transferred into a stainless-steel autoclave for the reaction at 180°C for 12 h. Finally, the products were washed with lots of water and dried in vacuum at 50°C for 24 h.

2.3 Fabrication of G/PTH/NG/GCE

The G/PTH/NG/GCE was fabricated by depositions of NG, guanine, and electropolymerization of thionine. Specifically, NG suspension (0.5 mg mL^{-1}) was sonicated

for 30 min prior to use. After sonication, 6 μL of NG suspension was dropped on the surface of a GCE and dried at room temperature. Then, the electrode was immersed into 4.4 mol L^{-1} acetic acid containing 0.5 mmol L^{-1} thionine solution and scanned from −0.4 to 1.4 V by cyclic voltammetry for 30 loops with the scanning rate at 80 mV s^{-1}. The electrode was washed with water, dried in air, and marked as PTH/NG/GCE. Finally, 6 μL of guanine solution (1.0 mg mL^{-1}) was dropped on to the surface of PTH/NG/GCE. After drying, the G/PTH/NG/GCE was obtained and kept for further use.

2.4 Electrochemical determination of antioxidant capacity

The antioxidant capacity assay was measured as our previous report with some modifications [25]. First, a certain concentration of test sample was mixed with freshly prepared Fe^{2+}–EDTA solution in phosphate buffer saline (PBS, 50 mmol L^{-1}, pH 3.5). Then, the fabricated G/PTH/NG/GCE was immersed into the mixture for 240 s. After incubation, the G/PTH/NG/GCE was washed with water and immersed in PBS (50 mmol L^{-1}, pH 1.5) to perform the SWV. The oxidation peak of guanine in SWV was recorded, and the measurements were completed three times consecutively to obtain the average values. The same amount of PBS was used as a control instead of samples. The antioxidant capacity of the sample was calculated using the following equation:

$$\text{Antioxidant capacity } \% = (I_s - I_c)/(I_0 - I_c) \times 100 \quad (1)$$

where I_0 is the oxidation peak current before damage in Fenton solution, and I_c and I_s are the oxidation peak currents of control and samples after damage in Fenton solution, respectively.

2.5 DPPH radical scavenging assay

The DPPH radical scavenging assay was measured according to Sridhar's report with some modifications [26]. Briefly, 2 mL of DPPH solution (0.05 mg/mL in methanol) and 1.0 mL of the sample with different concentrations were mixed. Then, the mixture was kept in dark for 5 min and transferred into a UV2700 UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan) for testing the absorbance at 517 nm. The same amount of water was used as a control. The scavenging rate of DPPH radicals was calculated by the following equation:

$$\text{DPPH radical scavenging rate } \% = (1 - A_s/A_0) \times 100\% \quad (2)$$

where A_s and A_0 are the absorbance of sample and control, respectively. The DPPH radical scavenging rate of sample was expressed as the concentration of sample needed to scavenge 50% of DPPH (EC_{50}).

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussions

3.1 Characterization of NG

TEM and SEM were used to characterize the morphology of prepared NG. The images of NG are shown in Figure 1. In the TEM image of NG (Figure 1a), the typical characteristics of NG such as smooth surface, sheet-like structure, and wrinkled edge could be found [27]. It could be seen the structure of NG was not influenced during nitrogen-doped procedure. The NG agglomerated into particles with nanoscale could be found in SEM image (Figure 1b). This kind of agglomeration might occur in the drying process of samples. The elemental mapping images of NG verified the existence of carbon and nitrogen elements in the materials (Figure 1c–e). In elemental mapping images, the green spots denoting nitrogen atoms and red spots denoting carbon atoms were well distributed over the NG [28]. It demonstrated that nitrogen atoms were uniformly doped in graphene, and the atomic percentage of nitrogen to carbon was about 5.59%. These characterization images could be considered as evidences that NG was successfully prepared in this research.

3.2 Fabrication of G/PTH/NG/GCE

The cyclic voltammograms for the electrochemical polymerization process of PTH on electrode with consecutive cyclic scanning are shown in Figure 2a. With the increasing scanning times, a pair of redox peaks could be found from 0 to 0.3 V, and the peak currents increased as well. Finally, a thin blue

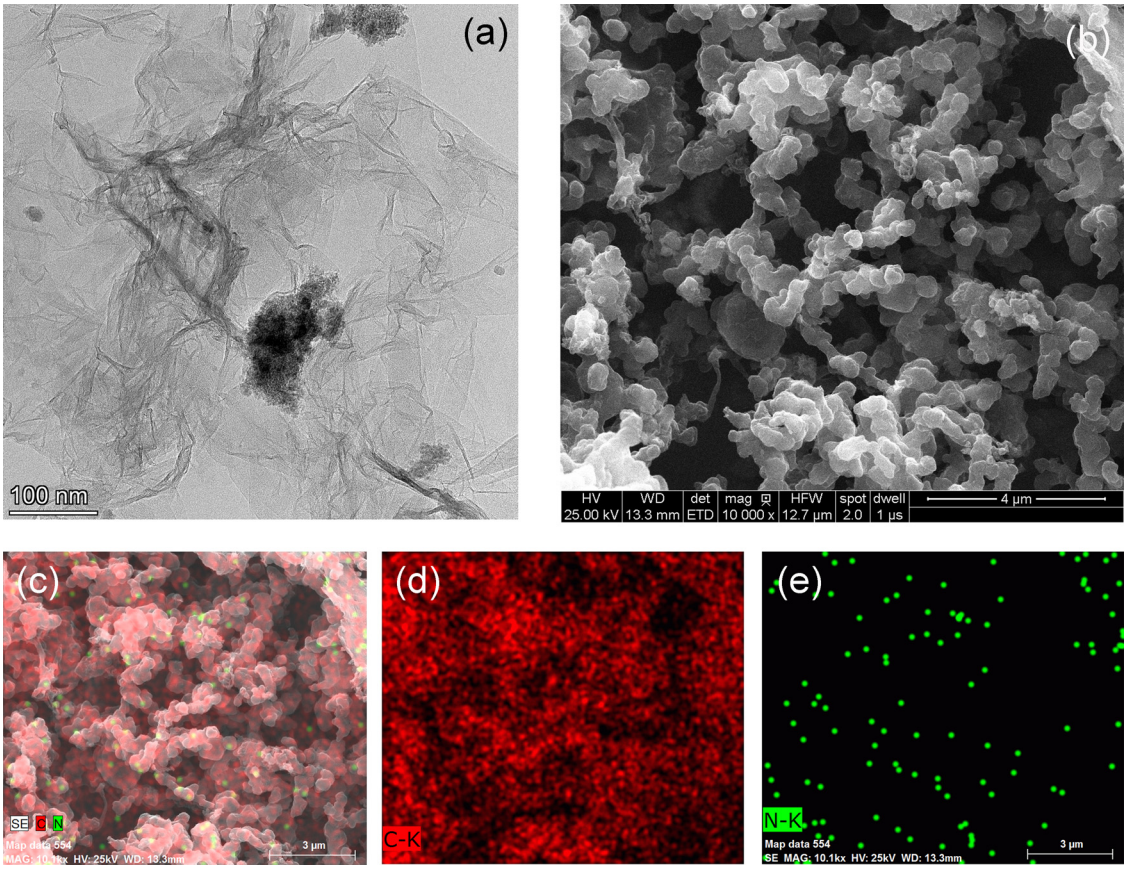


Figure 1: (a) TEM, (b) SEM, and (c–e) elemental mapping images of NG.

membrane could be observed on the surface of electrode, which showed that thionine was successfully polymerized on the electrode [29]. The experimental results showed that a stable and uniform blue PTH membrane could be obtained with a satisfied response through 30 loops consecutive cyclic scanning in 4.4 mol L^{-1} acetic acid containing 0.5 mmol L^{-1} thionine solution with a scanning potential range from −0.4 to 1.4 V and a scanning rate at 80 mV s^{-1}. The oxidation peak current of modified electrodes during the fabrication process was compared (Figure 2b). After the modifications of NG and PTH, the oxidation peak currents of electrodes were

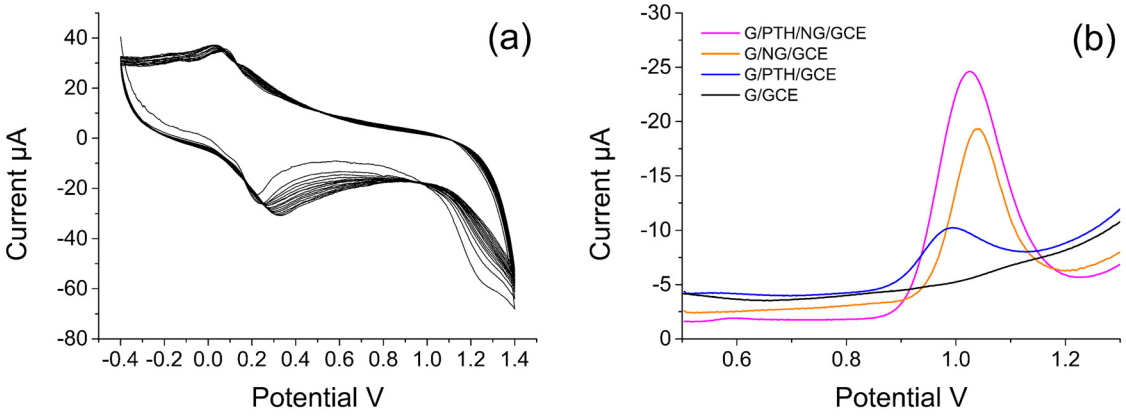


Figure 2: (a) The cyclic voltammograms for electrochemical polymerization of PTH on the electrode. (b) The oxidation peak current of several modified electrodes using SWV detection.

increased. Moreover, when NG and PTH were both modified on the electrode, the oxidation peak current was higher than that of electrode modified using any single material. It could be assumed that good electrical conductivities and electrocatalytic properties of NG and PTH played important roles in this sensor [30].

3.3 Optimization of fabrication and detection parameters

3.3.1 Effect of pH

The effect of pH on the oxidation peak of G/PTH/NG/GCE was studied. As shown in Figure 3a, with the growth of pH values (from 1.5 to 7.0), the peak currents decreased gradually and finally reached to a stable status. As a result, the peak current reached the maximum when pH was 1.5. Therefore, pH 1.5 was selected for electrolyte used in this study.

3.3.2 Effect of guanine concentration

The amount of guanine on the modified electrode could influence the voltammetric response (Figure 3b). As a result, the peak currents increased with guanine concentrations from 0.2 to 1.4 mg mL^{-1} and reached a stable state when guanine concentration was higher than 1.0 mg mL^{-1}. This behavior might be due to the fact that when the concentration of guanine reached a certain amount, the guanines adsorbed on the surface of electrode were saturated. More guanines could not be immobilized and were washed off during the washing procedure. Therefore, 1.0 mg mL^{-1} of guanine solution was selected as the optimum concentration of guanine solution for modification of the electrode.

3.3.3 Effect of Fe^{2+} concentration

It was reported that the concentration of Fe^{2+} affected the generation of hydroxyl radical and following damage to guanine [31]. The effect of Fe^{2+} concentration on the oxidation peak current of G/PTH/NG/GCE was studied. As shown in Figure 3c, the peak currents decreased with increasing of Fe^{2+} concentration. When the Fe^{2+} concentration was higher than about 2.5 mmol L^{-1}, there was only a little change of peak current and the damage to guanine reached the maximum. Therefore, 2.5 mmol L^{-1} of Fe^{2+} ions was chosen as the optimum Fe^{2+} concentration for this study.

3.3.4 Effect of incubation time

The effect of incubation time on oxidation peak current of G/PTH/NG/GCE was also investigated (Figure 3d). As the incubation time increased to 240 s, the peak currents decreased gradually. When the incubation time was longer than 240 s, the peak current remained constant, which showed the 240 s of incubation was enough for the damage to guanine. Therefore, 240 s incubation time was chosen as the optimum incubation time.

3.4 Determination of antioxidant capacity using G/PTH/NG/GCE

The fabricated G/PTH/NG/GCE was used in the detection of antioxidant capacity for investigated samples. As a well-known antioxidant, ascorbic acid was selected for the determination of antioxidant capacity using G/PTH/NG/GCE [32]. The prepared G/PTH/NG/GCE was processed with Fenton solution containing different concentrations of ascorbic acid and then detected using SWV. It was observed that the peak current increased with the growing concentration of ascorbic acid (Figure 4). A good linear relationship between peak current and ascorbic acid concentration could be found when the ascorbic acid concentration ranged from 0.5 to 3.0 mg L^{-1}. The corresponding linear function was fitted as I_p (μA) = $-7.7526 \times C_{AA} - 2.7169$ with a correlation coefficient at 0.999. The detection limit was 0.21 mg L^{-1} (S/N = 3).

To further evaluate the validity of determination for antioxidant capacity using G/PTH/NG/GCE, the samples containing ascorbic acid were measured by the standard addition method [33]. The analytical results are listed in Table 1. It could be found the ascorbic acid could be detected with a recovery rate of 98.7–101.5%, and the relative standard deviation (RSD) was less than 1.6%. These results illustrated that the determination of antioxidant capacity using G/PTH/NG/GCE was effective with good linearity and high accuracy.

3.5 The performances of G/PTH/NG/GCE

In order to evaluate the selectivity of G/PTH/NG/GCE in determination of antioxidant capacities, some samples

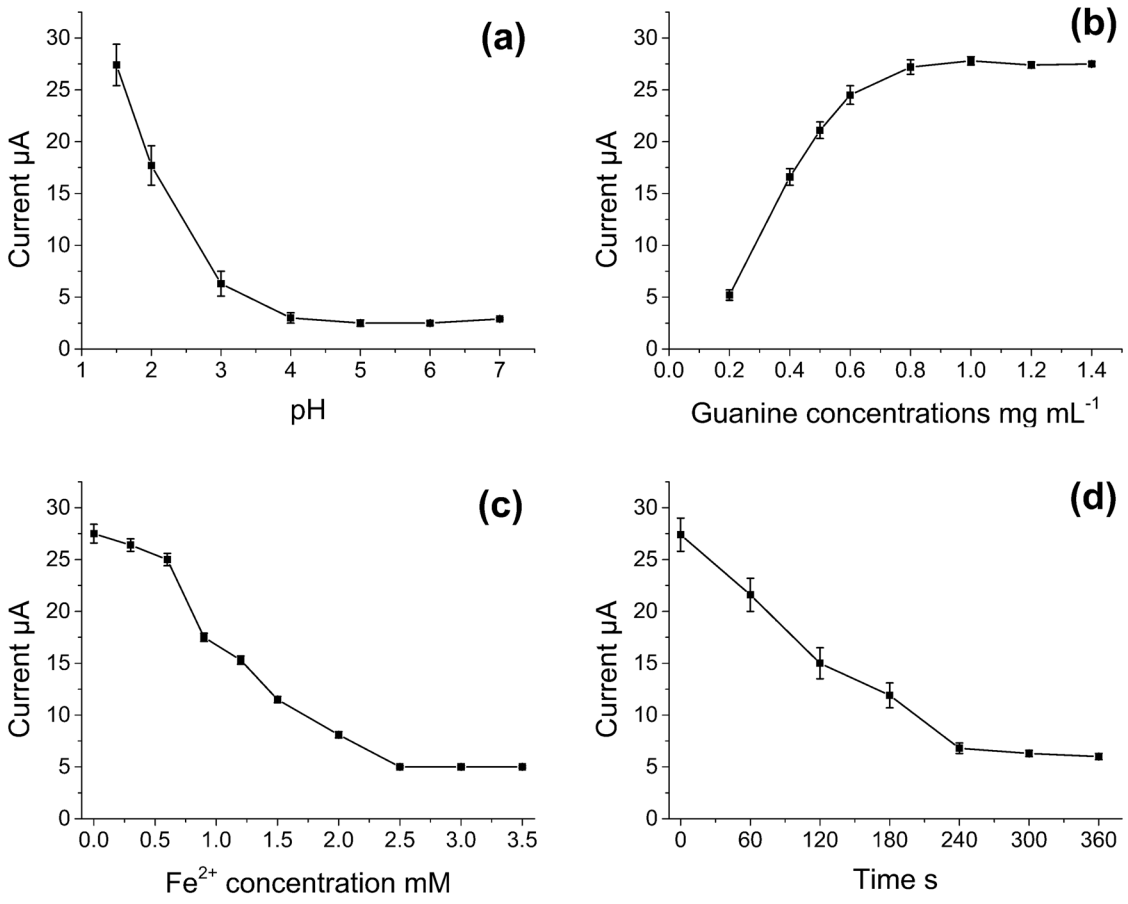


Figure 3: Effects of (a) pH, (b) guanine concentration, (c) Fe^{2+} concentration, and (d) incubation time on the oxidant peak currents of G/PTH/NG/GCE.

or substances were used for this comparison. Figure 5 shows the peak currents for these samples. It could be seen that the currents of substances, such as potassium chloride, sodium chloride, calcium chloride, and aluminum nitrate, were very low, which mean that these samples did not show antioxidant activities. However, the currents of ascorbic acid and galangin were relatively higher, which illustrated that these samples

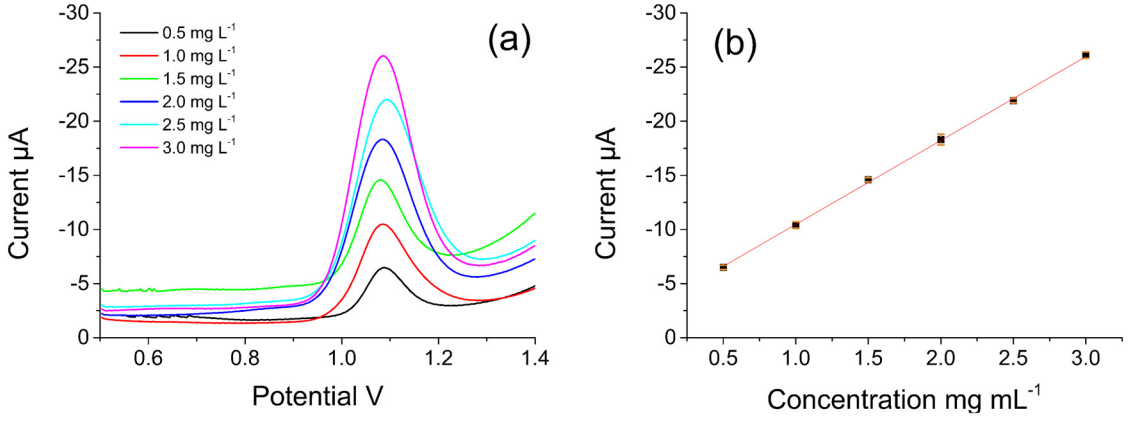


Figure 4: (a) The oxidation peak current of G/PTH/NG/GCE after incubation in Fenton solution with different concentration of ascorbic acid (a–i): 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}. (b) The calibration plot of oxidant peak current against the concentration of ascorbic acid.

Table 1: Determination of ascorbic acid at different addition (n = 5)

Samples	Concentrations (mg L^{-1})	Added (mg L^{-1})	Found (mg L^{-1})	Recovery (%)	RSD (%)
Ascorbic acid	2.0	0.25	2.28	101.5	0.91
	2.0	0.5	2.47	98.7	0.47
	2.0	1.0	2.98	99.2	1.59

have antioxidant capacities. The antioxidant capacities of ascorbic acid and galangin would be verified in the following study. This comparison showed that the determination of antioxidant capacity using G/PTH/NG/GCE has a good selectivity of antioxidants and showed a certain anti-interference ability.

The reproducibility and stability of G/PTH/NG/GCE were evaluated as well. Several electrodes were used for parallel measurements to evaluate the reproducibility of test. The results showed a good reproducibility in 10 times tests with the RSD value at 3.1% (shown in Figure 6a). After one week of storage at 25°C for a certain electrode, the peak current reduced about 5.2% (shown in Figure 6b). These results demonstrated that the modified electrode was stable for determination at least for one week.

3.6 Comparison of antioxidant capacity using G/PTH/NG/GCE and DPPH method

The antioxidant capacities of three flavonoids were analyzed by fabricated G/PTH/NG/GCE. The concentration of three flavonoids was unified at 6.0 μmol L^{-1}, and the results of the three flavonoids and ascorbic acid are listed in Table 2. Ascorbic acid displayed the highest antioxidant capacity (80.4 ± 2.5%), then followed by myricetin (73.1 ± 3.7%), kaempferol (63.2 ± 1.2%), and galangin (60.7 ± 3.1%). Simultaneously, the antioxidant capacities of myricetin, kaempferol, and galangin were detected by the DPPH assay, and the activities were presented as EC_{50} values in Table 2. For the DPPH assay, the lower EC_{50} values mean higher antioxidant capacities of the sample [34]. The rank of three flavonoids from high antioxidant activity to low was myricetin, kaempferol, and galangin. These results were in accordance with that obtained through the G/PTH/NG/GCE method.

As reported in references, the antioxidant activities of flavonoids were affected by structure and substituent groups [35–37]. The main structure of myricetin, kaempferol, and galangin was the same with different hydroxyl groups on B ring (Figure 7). The number of hydroxyl groups on B ring mostly affected the antioxidant activities of flavonoids, and more hydroxyl groups on B ring showed higher antioxidant activities [38]. According to this principle, myricetin should have the best antioxidant ability among these three flavonoids, while galangin would have the lowest antioxidant capacity. The antioxidant capacities detected by G/PTH/NG/GCE and DPPH methods confirmed this principle, which showed that this kind of detection followed reported principle and could be considered effective in the detection of antioxidant capacities.

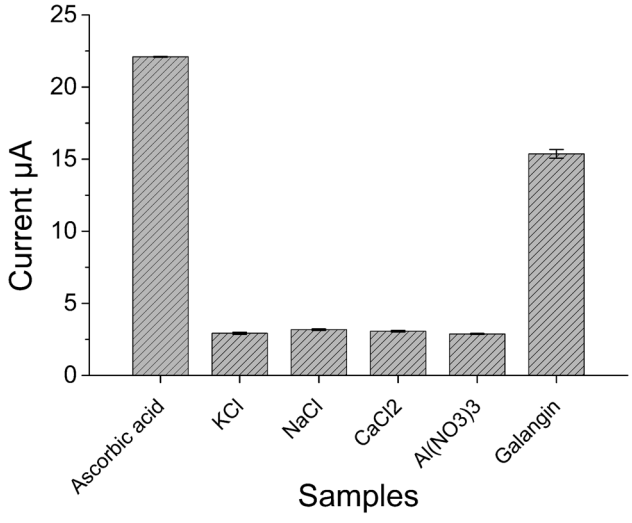


Figure 5: The selectivity of G/PTH/NG/GCE in the detection of samples (KCl, NaCl, $CaCl_2$, $Al(NO_3)_3$ and galangin: 5.0 μmol L^{-1}).

3.7 Antioxidant capacities of real samples

In order to explore the application of G/PTH/NG/GCE for real samples, the prepared G/PTH/NG/GCE was utilized to evaluate the antioxidant capacities of fruit juices and plant extracts. Three kinds of fruit juices including grape juice, guava juice, and orange juice were bought from the local supermarket. Three plant extracts were made in our labs including jute leaves extract, ramie leaves extract, and hemp leaves extract. All of the samples were filtered to remove insoluble substances and set the

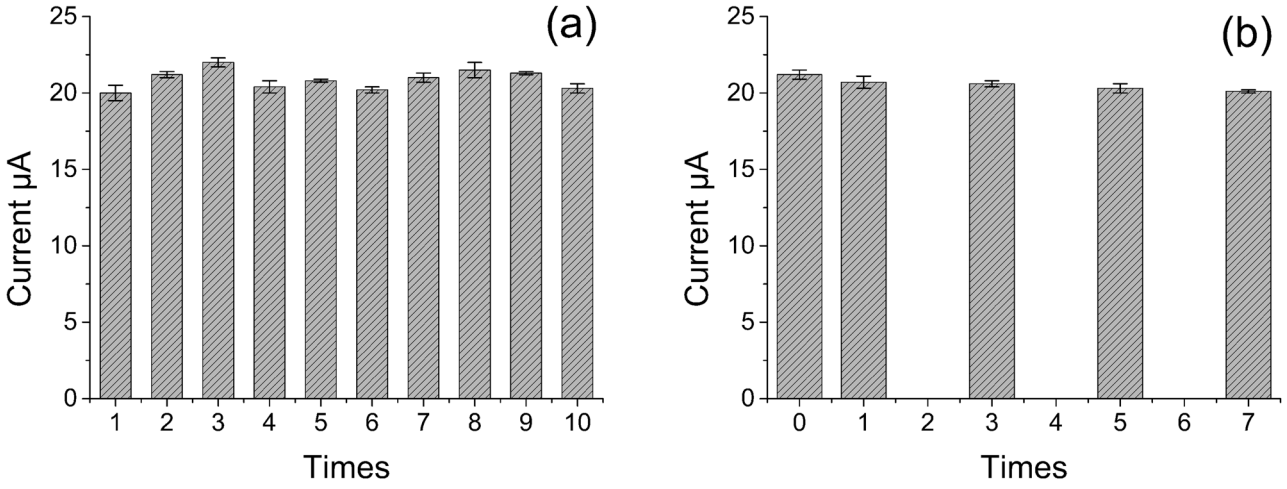
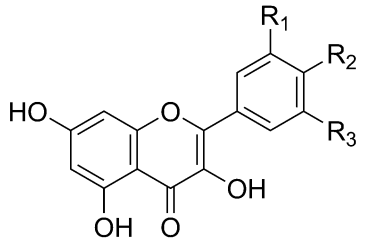


Figure 6: The (a) reproducibility and (b) storage stability of G/PTH/NG/GCE in the detection of ascorbic acid.

Table 2: Determination of antioxidant capacities of myricetin, kaempferol, and galangin compared to ascorbic acid using G/PTH/NG/GCE and DPPH method

Flavonoids	Antioxidant capacity	
	G/PTH/NG/GCE (%)	DPPH method (μmol L^{-1})
Myricetin	73.1 ± 3.7	10.5 ± 0.4
Kaempferol	63.2 ± 1.2	23.5 ± 1.8
Galangin	60.7 ± 3.1	28.4 ± 2.4
Ascorbic acid	80.4 ± 2.5	5.5 ± 0.5



	R_1	R_2	R_3
Myricetin	OH	OH	OH
Kaempferol	H	OH	H
Galangin	H	H	H

Figure 7: The chemical structures of myricetin, kaempferol, and galangin.

concentration at 2.0 mg L^{-1}. As shown in Figure 8, all of the three juices showed antioxidant capacities from 56% to 68%, whereas the plant extracts showed relatively higher antioxidant capacities (from 85% to 92%) than those of juices. It might be due to fact that the plant extracts contain more active compounds and antioxidants. Based on these results, the fabricated G/PTH/NG/GCE showed predictable ability in determination of antioxidant capacities of real samples.

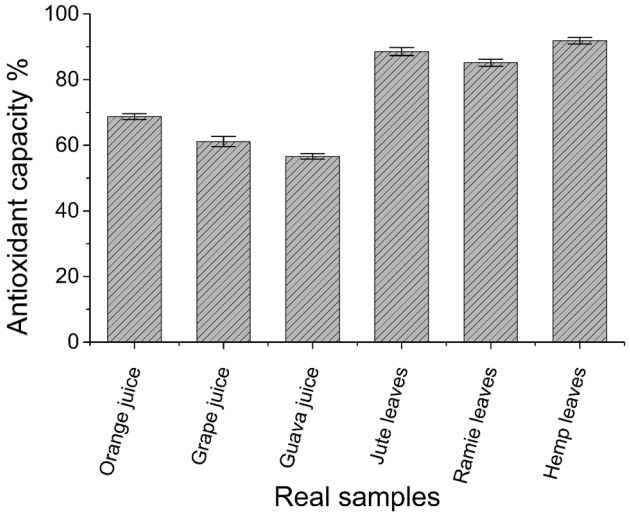


Figure 8: Determination of antioxidant capacities of fruit juices and plant extracts using G/PTH/NG/GCE.

4 Conclusions

In this research, the G/PTH/NG/GCE was fabricated and used for the electrochemical determination of antioxidant capacity. After the modification, the related oxidation peak current on G/PTH/NG/GCE was improved. The factors including pH, guanine concentration, Fe^{2+} concentration, and incubation time were optimized for the optimum performance of the modified electrode. The G/PTH/NG/GCE showed good linearity

and selectivity, satisfied reproducibility, and storage stability. Three flavonoids were selected as the samples for the antioxidant capacity tests using G/PTH/NG/GCE and DPPH methods, and the results have a good agreement. Some fruit juices and plant extracts were also tested as real samples. The proposed G/PTH/NG/GCE provided to be a rapid and efficient sensor for the evaluation of antioxidants in food and natural chemical fields.

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Conflict of Interest: The authors declare no conflict of interest.

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