

Evaluation of Total Antioxidant Activity of Different Floral Sources of Honeys Using Crosslinked Hydrogels

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Antioxidants in traditional food can protect organisms from free radicals and prevent the occurrence and development of cancer, cardiovascular and cerebrovascular diseases and diabetes mellitus. The accurate and quick evaluation of total antioxidant activity (TAC) in food samples remains a challenge. In this work, a zinc ion-crosslinked chitosan hydrogel was prepared to evaluate the TAC of honey from different floral sources. After optimization, locust honey, linden honey, jujube honey, wattle honey, citrus honey and Chinese milk vetch honey were used as experimental samples. The results indicate that Chinese milk vetch honey exhibits the highest TAC of the honeys from the studied floral sources.

Keywords: Electrochemistry; Total antioxidant activity; Hydrogel; Honey; Metal ions

1. INTRODUCTION

In the 1940s, Shell Companies physicist Denham Harman tried to determine the mechanism of ageing. In 1956, he published the famous paper, “Ageing: A theory based on free radical and radiation chemistry” [1]. He believed that the ageing of organisms was caused by the accumulation of reactive oxygen species (ROS) produced by cells in tissues. He further put forward the theory that antioxidants can slow down ageing [2]. Organisms themselves have a complex system of antioxidant metabolites and enzymes. By synergistic action between antioxidant metabolic intermediates, products and enzymes,

important cellular components, such as DNA, proteins and lipids, are protected from oxidative damage. However, an imbalance between ROS components and the antioxidant system in organisms can lead to oxidative stress, which is considered to be a cause of many diseases such as rheumatoid arthritis and Parkinson's disease [3,4]. Therefore, in addition to various other health measures, taking antioxidants and eating fruits and vegetables rich in natural antioxidants are considered to delay ageing [5]. Total antioxidant capacity (TAC) has become an important index to measure the antioxidant capacity of food, fruit juice and additives.

Honey is a sweet substance stored in honeycomb by bees, which mix nectar collected from plants, exuded from plant cells or excreted by insects with the secretion of bee salivary glands. Honey not only is a healthy food with high nutritional value but also has a variety of biological activities [6-8]. Honey has long been used as food, but its antioxidant function has only recently been discovered. The antioxidant strength of honey depends on the source of the honey, season, environmental factors, and the process of honey production [9,10]. Antioxidant components in honey include flavonoids and phenolic acids, enzymes (glucose oxidase, catalase), ascorbic acid, Maillard reaction products, carotenoids, organic acids, amino acids and proteins. These natural antioxidants have a wide range of biological activities such as antibacterial, anti-inflammatory, anti-allergic, anti-thrombosis and vasodilation functions [11,12].

Recently, we fabricated a cross-linked hydrogel based on low-molecular weight chitosan and metal ions [13,14]. The proposed hydrogel shows a very sensitive response towards ROS and is capable of measuring TAC. In this work, we attempted to use a zinc ion-crosslinked hydrogel for TAC evaluation of different types of honey. After optimization, the hydrogel was successfully used to measure the TAC of honey. The proposed method shows great potential for TAC analysis of food samples.

2. EXPERIMENTAL

2.1. Materials

Silver nitrite, chitosan (deacetylation: 75%–80%, 50000–190000 Da), acetic acid, hydrogen peroxide and potassium ferricyanide were all analytical grade. Honey from different floral sources, including locust honey (Tongren Tang), linden honey (Tongren Tang), jujube honey (Tongren Tang), wattle honey (Cisheng Tang), citrus honey (Baochun) and Chinese milk vetch honey (Guansheng Yuan), were purchased from online shops. Acetic acid was used as the electrode during measurement.

2.2. Hydrogel fabrication

The synthesis of metal ion-crosslinked hydrogels was performed according to our previous method with some modifications [13,14]. Specifically, zinc ions were selected as crosslinking ions for hydrogel preparation. First, chitosan was dissolved into acetic acid to form a 1% solution using sonication. Then, zinc acetate was dissolved into the chitosan solution at a concentration of 2 mM using vigorous stirring. Then, 0.1 M NaOH was slowly added dropwise into the above solution until a gelation

phenomenon was initiated. The formed hydrogel was stored in a refrigerator before TAC measurement and could be used over a month without disintegration.

2.3. TAC measurement

The TAC measurement was conducted using an electrochemical analyser (CHI 760E) using a three-electrode system. A glassy carbon electrode, a Pt wire electrode and a Ag/AgCl (3 M) electrode were used as the working electrode, counter electrode and reference electrode, respectively. During the measurement, three electrodes were inserted into the hydrogel to form a circuit. Then, Fenton solution was introduced into the hydrogel to initiate depolymerization. Honey solution was subsequently added into the hydrogel to scavenge ROS. Cyclic voltammetry (CV) was used for analyzing the change in signal from metal ions before and after the addition of honey to evaluate the TAC of honey from different floral sources.

3. RESULTS AND DISCUSSION

Hydrogels are three-dimensional network polymers. Because of their strong hydrophilic groups, they swell in water and can absorb a large amount of water without dissolving [15-18]. Hydrogels can be classified into natural hydrogels and synthetic hydrogels. The structures and properties of hydrogels made from natural polysaccharides such as chitosan, sodium alginate, agar and carrageenan are slightly less controllable than those of synthetic hydrogels, but due to their excellent biological safety and biodegradability, natural hydrogels have shown broad application prospects in the field of biomedicine [19-22]. For example, a stretchable and compressible flexible hydrogel electrolyte with high ionic conductivity was recently prepared using protonated polyacrylamide as the main polymer and olefin-based hybrid silica nanoparticles as the crosslinking agent [23]. Inspired by the use of hydrogels as electrolytes in the field of supercapacitors, we believe that hydrogels can not only be used for surface modification of electrodes but also can serve as an excellent electrolyte environment in the field of electrochemical analysis and detection. Based on the characteristics of intelligent hydrogels, a natural polymer-metal ion-crosslinked hydrogel has been developed for use in a new electrochemical detection method. In this method, the hydrogel is used not only as an electrolyte but also as an electrochemical probe for quantifying the intensity of external stimuli induced by hydrogels.

Figure 1A shows the scanning electron microscope (SEM) image of a zinc ion-crosslinked chitosan hydrogel. The morphology of the hydrogel shows a typical 3D porous structure. Figure 1B shows the CV profiles of the chitosan solution and the hydrogel. No characteristic signals are observed for the chitosan solution, suggesting that the chitosan shows no redox reaction in the scan range. On the other hand, the hydrogel shows a distinct oxidation peak centred at -0.6 V arising from the oxidation of metallic zinc. Since the peak arising from the oxidation of zinc is more distinct than that from the reduction of zinc, this signal has been selected for estimating the TAC of honey from different floral sources.

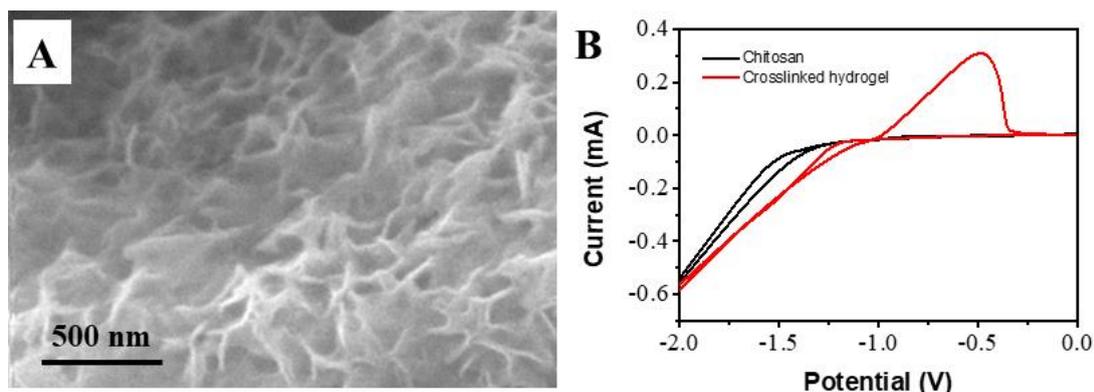


Figure 1. (A) SEM image of the zinc ion-crosslinked chitosan hydrogel. (B) CV profiles of the chitosan solution and the hydrogel.

Currently, the conventional methods to detect TAC include the oxygen free radical absorptive capacity test, the total oxidant scavenging capacity test [24], the ROO-induced beta-carotene fading method, the iron reduction capacity test and the Folin-Ciocalteu colorimetric method [25-29]. These optical detection methods have some insurmountable shortcomings such as the need for longer detection times, the need to decolorize the samples in advance and the absence of anti-interference capability. In contrast, the electrochemical method for TAC detection has become an exciting alternative method because it is simple, highly sensitive and portable [26,30-35]. Before testing the TAC of honey from different floral sources, a hydrogel assay needed to be optimized.

Figure 2A shows the CV profiles of the proposed hydrogel before and after the addition of various concentrations of Fenton solution. The redox peak current from the zinc ions showed a positive relationship with the Fenton solution concentration. Hydroxyl radicals can induce depolymerization of the chitosan hydrogel. Upon the injection of Fenton solution, unstable hydroperoxide anions are produced that decompose to form reactive hydroxyl radicals ($\bullet\text{HO}$) [36]. The rupture of glycosidic bonds caused by the hydrogen abstraction reaction between the hydroxyl radical and chitosan polysaccharide chains causes the depolymerization process [37]. This phenomenon can be ascribed to the depolymerization of the hydrogel due to the ROS produced by the Fenton solution. The ROS could attack the bonds in chitosan, breaking them and consequently freeing the metal ions from the linkage. A clear liquid was observed when a high concentration of hydrogen peroxide was injected into the chitosan hydrogel. Therefore, more metal ions can move to the surface of the electrode and participate in the redox reaction. A large oxidation current difference before and after the addition of Fenton solution is beneficial for the analysis of TAC. Therefore, two parameters have been studied for optimization. Hydroxyl radicals are one of the most common ROS and are primarily responsible for cellular disorders and cytotoxic effects. Chitosan is constructed of glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) copolymers linked by beta-1,4-glycosidic bonds. Under ideal conditions, depolymerization of chitosan should release both GlcN and GlcNAc. Figure 2B shows the effect of zinc ion concentration on the oxidation performance. As shown in the figure, the difference in current before and after the addition of Fenton solution increases as the zinc ion concentration increases from 0.01 to 0.2 mM. Further increasing the zinc ion concentration results in a decrease in the difference between the current measured

before or after the addition of Fenton solution. Therefore, a zinc ion concentration of 2 mM was selected for hydrogel formation. Figure 2C shows the effect of the hydrogel depolymerization time on the difference in current before and after the addition of Fenton solution. The difference in current shows a significant increase after the initiation of polymerization. After 4 min, the increase in the current difference achieves saturation. Therefore, a 4 min depolymerization time was selected for further study.

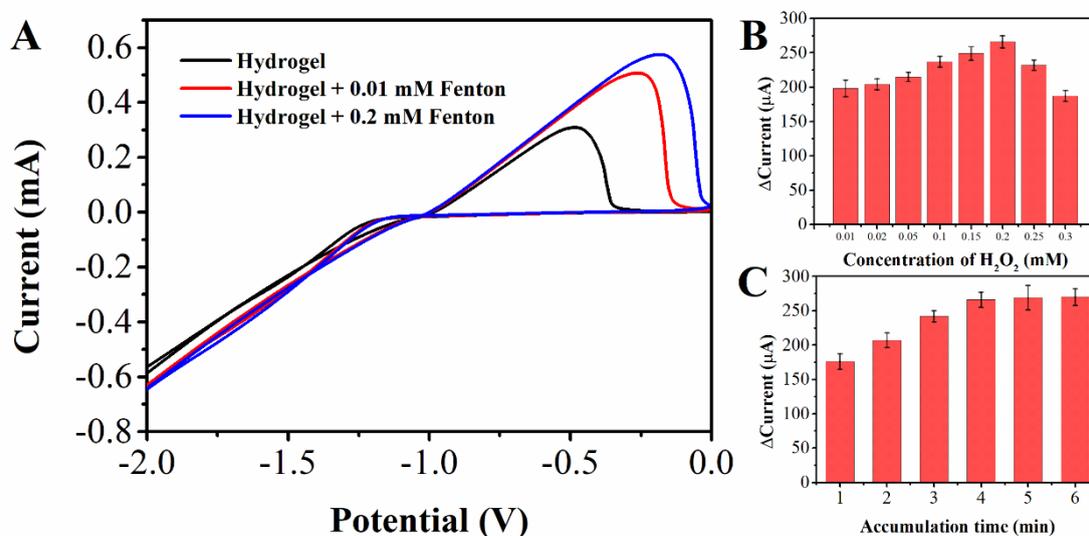


Figure 2. (A) CV profiles of the hydrogel before and after the addition of Fenton solution. (B) Effect of zinc ion concentration on the oxidation current difference before and after the addition of Fenton solution. (C) Effect of the depolymerization time on the oxidation current difference before and after the addition of Fenton solution.

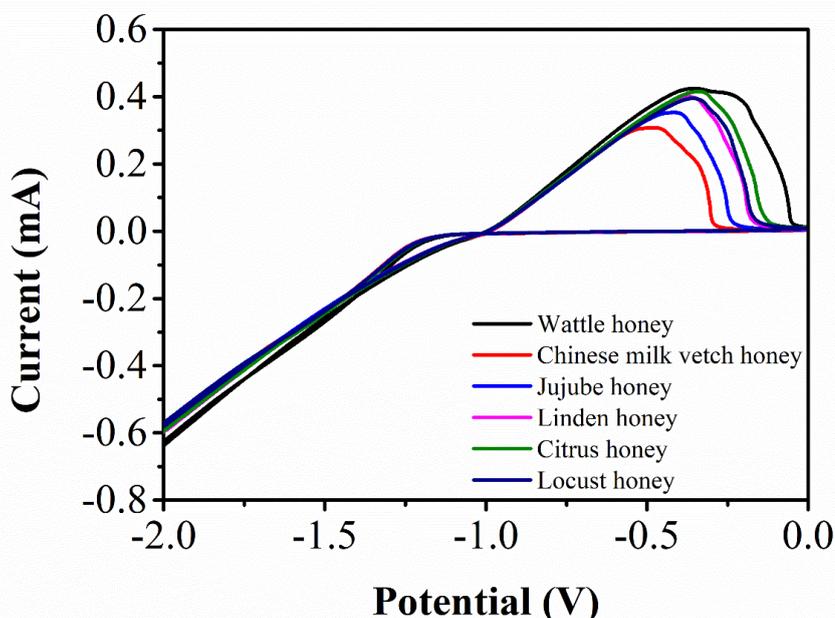


Figure 3. CV profiles of hydrogel after the addition of 0.2 mM Fenton solution and 0.1 mL of honey solution (locust honey, linden honey, jujube honey, wattle honey, citrus honey and Chinese milk vetch honey).

Crosslinked hydrogel was then applied as an electrochemical detection platform for TAC analysis of honey from different floral sources. During the depolymerization process, different honey solutions were added regularly to suppress the ROS-induced depolymerization of the hydrogel. The total number of metal ions that diffuse to the surface of the electrode are reduced due to the decreased degradation of chitosan and consequently result in a difference in electrochemical signal. Different honey TAC measurements were evaluated by signal comparison. Then, we studied and optimized the stability and reliability of the electrochemical TAC detection method based on hydrogel depolymerization.

Hydroxyl radicals are highly reactive and occur naturally in cells and extracellular fluids but can be deactivated in the presence of antioxidants [38,39]. Figure 3 shows the CV profiles of the hydrogel after the addition of 0.2 mM Fenton solution with 0.1 mL of honey aqueous solution (0.5 mg/mL). As shown in the figure, the oxidation peak currents of zinc oxidation in solutions containing honey are smaller than that observed for the control group, suggesting that all types of honey indeed display antioxidant activity to scavenge ROS. In addition, honey from different floral sources show different TAC during the assay.

To verify the TAC for honey from different sources, the differential pulse voltammetry (DPV) method has been used due to its high sensitivity. Figure 4 shows DPV profiles indicative of the TAC of locust honey, linden honey, jujube honey, wattle honey, citrus honey and Chinese milk vetch honey. It can be clearly seen that the wattle honey shows the highest peak current, suggesting its TAC is relatively lower than the TAC of honey from other sources. Among the honeys from different floral sources, the Chinese milk vetch honey exhibited the lowest peak current, suggesting that its high TAC could suppress the ROS and lower the signal from zinc oxidation. Based on the results, the TAC of honey from different floral sources follows the following order: Chinese milk vetch honey > jujube honey > linden honey > locust honey > citrus honey > wattle honey.

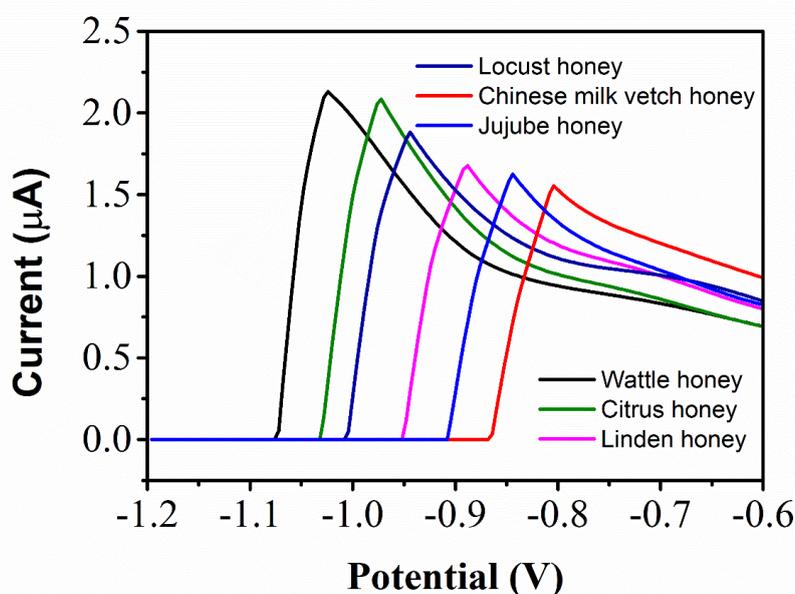


Figure 4. DPV profiles of hydrogel after the addition of 0.2 mM Fenton solution and 0.1 mL of honey solution (locust honey, linden honey, jujube honey, wattle honey, citrus honey and Chinese milk vetch honey).

The reproducibility of the TAC evaluation platform is very important for practical applications. Therefore, we produced five independent hydrogels for Chinese milk vetch honey TAC analysis. As shown in Figure 5A, four hydrogels show very similar profiles, with a relative standard deviation (RSD) of 4.5%. Therefore, the hydrogel displayed excellent reproducibility for TAC analysis. Figure 5B shows four hydrogels used for Chinese milk vetch honey TAC analysis after different storage times. The prepared hydrogel shows consistent performance over one month.

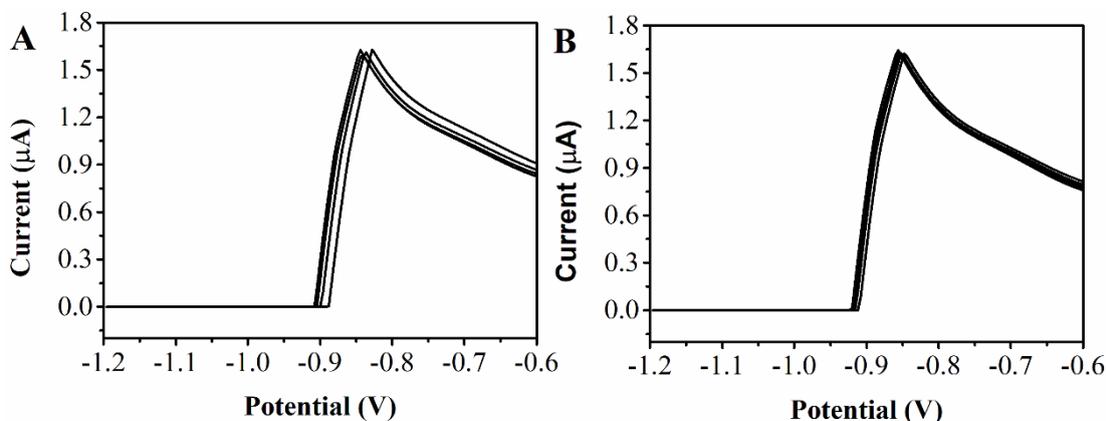


Figure 5. (A) DPV profiles of four hydrogels after the addition of 0.2 mM Fenton solution and 0.1 mL of Chinese milk vetch honey. (B) DPV profiles of four hydrogels stored over 4 weeks after the addition of 0.2 mM Fenton solution and 0.1 mL of Chinese milk vetch honey.

4. CONCLUSION

In conclusion, a zinc ion-crosslinked hydrogel was prepared by adding zinc acetate into chitosan solution while carefully adjusting the pH. The redox activity of zinc in the hydrogel can be used as an effective probe for evaluating the TAC of honey from different floral sources. In this work, locust honey, linden honey, jujube honey, wattle honey, citrus honey and Chinese milk vetch honey were selected as “real-world” samples. The results indicate that the TAC of the honey from different sources follows the following order: Chinese milk vetch honey > jujube honey > linden honey > locust honey > citrus honey > wattle honey.

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