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A Simple Electrochemical Method to Detect Tetracycline in Honey Samples using Electrochemically Activated Glassy Carbon Electrode in Surfactant Medium

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Abstract- An electrochemical method for the determination of tetracycline has been reported, employing cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques with an activated glassy carbon electrode serving as the working electrode. In the presence of a suitable surfactant, the peak current enhancement of tetracycline was achieved. The interference effects and their elimination of the electrochemical method were also studied in the presence of various ions. The current responses obtained under the optimum experimental conditions through the DPV technique of tetracycline increased linearly with concentrations in linear dynamic ranges of 1.0 – 10 μmol L^{-1} and 10 – 100 μmol L^{-1} with 0.31 μmol L^{-1} detection limits. Combined with solid-phase extraction (SPE), the proposed method was effectively used for the determination of tetracycline residues in honey, achieving recovery rates ranging from 81.8% to 91.8%. The results suggest that this method is applicable for the detection of TC in complex food matrices.

Keywords- Tetracycline; Honey; Voltammetry; Glassy carbon electrode; Solid-phase extraction

1. INTRODUCTION

Honey, a nutrient-rich and valuable natural product derived from bees, is consumed by people all over the world. It has high commercial value due to the presence of about 200 compounds, including sugars and complex mixtures of polyphenols, amino acids, proteins, saccharides, peptides, enzymes, organic acids, carotenoids, vitamins, and minerals [1-4]. Honeybees can be affected by pests and diseases that lead to a decline in bee populations and honey production. Two of the most significant bacterial diseases impacting honeybee larvae are European foulbrood and American foulbrood. These diseases are caused by the bacterium *Melissococcus plutonius* and the spore-forming bacterium *Paenibacillus larvae*, respectively [5-7]. American foulbrood is highly contagious and spreads through adult bees, swarming, drifting, foraging, or contaminated equipment. European foulbrood similarly affects larvae, leading to discoloration and premature death. Although European foulbrood is less contagious and does not produce long-lasting spores, its transmission pathways are comparable to those of American foulbrood [5]. Since 1940, tetracycline (TC), as a broad-spectrum antibiotic, has been employed in farming to prevent and combat diseases in honeybees [8, 9]. Owing to its low cost and widespread availability, TC is frequently employed worldwide against bacterial diseases in bees. It is also added to honey products to preserve their marketable state [9, 10]. Consequently, TC may be present in honey and its commercial products. Long-term consumption of honey products contaminated in this way may result in adverse effects on humans, including digestive disorders, allergies, and impaired tooth and bone development [10, 11]. For this reason, several countries have introduced and enforced regulations that promote the adoption of best beekeeping and manufacturing practices to ensure the safe production and distribution of honey products. According to regulations within the European Union, no maximum residue limit (MRL) has been established for this antibiotic in honey. This means that TC residues in honey is not legally permissible for commercialization in European countries. The allowable concentration of TC in honey is set at 15 μg kg^{-1} in France, while in the United Kingdom, the MRL is 50 μg kg^{-1}. In Korea, MRL for TC in honey is 300 μg kg^{-1}, whereas in China, it is 50 μg kg^{-1} [12-14].

For years, traditional techniques have been developed to separate and determine TC residues accurately and precisely, including capillary electrophoresis (CE), liquid chromatography (LC), and immunoaffinity method. A CE method has been developed for the quantification of TC in fish muscle samples. This method employs extended light-path silica capillaries and low-wavelength UV detection to enhance sensitivity, achieving good linearity, sensitivity, and selectivity. The limit of detection for TC residues ranged from 1.3 to 1.8 ng g^{-1}, with quantification limits between 4.3 and 5.9 ng g^{-1}, and a recovery rate of 84.0% [15]. Gustavo et al. developed a HPLC method with fluorescence detection for determination of TC and other antibiotic residues in honeybee. This method demonstrated excellent linearity, with an accuracy range of 86% to 111%, and a limit of detection of 8 μg kg^{-1} [16]. Furthermore, an

optimized approach for detecting tetracyclines in honey involves chelation with metal ions, solid-phase extraction (SPE), and LC separation on a C18 column. TC residues were quantified using LC with UV detection at 355 nm, achieving detection limits of 5-10 ng g^{-1} and quantification limits of 15 ng g^{-1}. LC-MS/MS was employed for confirmation, with detection limits ranging from 1-2 ng g^{-1}. This method provides high recovery and low variability, rendering it suitable for routine residue analysis in honey [17]. Additionally, a sensitive, wash-free immunoassay utilizing a Eu^{3+} cryptate complex for fluorescence detection has been developed for the determination of TC residues in environmental samples. The assay achieves a low limit of detection (0.0106 ng mL^{-1}), exhibits a linear range from 0.0273 to 9.2645 ng mL^{-1}. Results can be obtained in as little as 30 minutes, with recovery rates ranging from 84.3% to 107.2% [18]. However, despite the effectiveness of these methods, they often require expensive laboratory instruments, long detection times, and highly trained technicians, making them costly and less accessible for routine use. Therefore, a rapid, simple, and real-time method for detecting TC in food of animal origin and environmental samples is urgently needed.

Recently, electrochemical analytical methods have been progressively researched and developed to quantify the content of TC in many sample matrices, ranging from simple to complex [19-22]. They are known for their simple operation, fast response, low detection limit, low sample volume required, and low analysis costs, which have been successfully applied for TC detection in honey samples [19, 20]. Madiha and colleagues employed molecularly imprinted polymers and gold nanoparticles to modify a gold electrode, thereby improving the sensitivity and selectivity of the method for TC detection in honey samples [19]. The method demonstrated detection ranges from 224 fmol L^{-1} to 22 nmol L^{-1} with a detection limit of 222 pmol L^{-1}. Additionally, a gold electrode modified with an antimony film was employed for the determination of TC in the same matrix [20]. The detection of TC was linear over 0.40 – 3.00 μmol L^{-1} concentration range with 0.15 μmol L^{-1} limit of detection and recovery rate ranging from 91.5 to 109.7%. A highly efficient electrochemical aptamer sensor (SnC@Au@Apta) for TC detection was also developed. Metal tin provides high conductivity, while electrospun nanofibers enhance sensitivity. Gold nanoparticles on SnC improve performance and act as binding sites for the TC aptamer. The sensor shows a wide detection range (0.001–100 μmol L^{-1}), low limit of detection (0.83 nmol L^{-1}), and excellent selectivity, stability, and reproducibility [23]. However, a significant limitation of these methods lies in the labor-intensive nature of electrode surface modification, as well as the use of costly electrodes and reagents, which may hinder their applicability for routine quality control. This study aims to further develop a simple and cost-effective electrochemical analytical method using a glassy carbon electrode (GCE) for detecting TC residues in Vietnamese honey samples. Several surfactants were used as a strategy to enhance the preconcentration of the target analyte on GCE surface. Additionally, the effects of various cations such as NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Al^{3+} on the signal of TC and their elimination were carefully studied.

2. EXPERIMENTAL SECTION

2.1. Chemicals and reagents

TC (96%) was sourced from the Institute of Drug Quality Control (Ho Chi Minh city, Vietnam). A standard solution was prepared by dissolving in ethanol (99.5%, Fisher). The stock solution was stored at 5 °C. Working solutions were prepared using serial dilution daily.

All other chemicals used in this study were of analytical-reagent grade, and their solutions were prepared using deionized water. Sodium sulfate 0.2 M solutions at pH 3.0 were prepared by dissolving 2.842 g Na_2SO_4 in 100 mL deionized water, then adding 1.00 mL of 0.05 M H_2SO_4 and mixing well. The pH was adjusted to 3.0 using 1 M H_2SO_4 or 1 M NaOH. This solution was used as a supporting electrolyte for electrochemical measurements.

Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Al^{3+} were used from 1000 mg L^{-1} stock solution (Merck). Ammonium chloride (NH_4Cl) was obtained from Sigma at 98.98% purity.

The surfactants tested were nonionic type, triton X-100 (99%, Sigma), the anionic surfactant sodium dodecyl sulfate (SDS, 90%, Merck), and the cationic surfactant cetyltrimethylammonium bromide (CTAB, 99%, Sigma).

2.2. Apparatus

Voltammetric measurements were conducted using an OrigaStat - OGS080 (Origalys, France) controlled by OrigaMater5 software (version 2.3.0.4). Cyclic voltammetry (CV) was employed in the GCE surface activation step and differential pulse voltammetry (DPV) was used to quantify TC in solutions. All measurements were conducted in an electrochemical cell maintained at 25^{o}C. The cell configuration included a GCE working electrode (3.0 mm diameter) from ALS (Japan), a platinum wire auxiliary electrode (1.0 mm diameter), and a calomel reference electrode immersed in 3 M KCl solution.

2.3. Voltammetric measurements

For the pretreatment of the GCE surface, a polishing step with alumina powder 0.5 μm was performed, followed by sonication in ethanol and deionized water for three minutes. After that, the GCE electrode was activated by scanning CV in 0.5 M H_2SO_4 from -0.20 to 1.50 V for 20 continuous cycles at a scan rate of 100 mV s^{-1}. After the surface treatment step, the electrode was ready for use.

For quantification of TC, the electrodes were immersed in the electrochemical cell containing a solution of TC and 0.2 M Na_2SO_4 at pH 3.0 with surfactants. The adsorption process was then carried out for 8 min under a magnetic stirrer at 1600 rpm. After the adsorption step, the solution was allowed to stabilize for 5 seconds prior to measurements. DPV was conducted from +0.60 to +1.20 V at a scan rate of 50 mV s^{-1} and a pulse amplitude of 100 mV. All measurements were repeated in triplicate at room temperature.

2.4. Pretreatment of the honey samples

Honey samples were purchased from the local market. one gram honey sample was weighed into a 50 mL centrifuge tube. At this step, any spiking solutions were added, and the spiked sample was left at ambient temperature for at least 5 h. Next, 20 mL of 0.5 M phosphate buffer was added and vortexed well until a homogeneous solution was obtained. The diluted sample was loaded onto a solid phase extraction (SPE) cartridge (Agilent, C18, 6 mL) at a flow rate of 2 mL min^{-1}. The cartridge was preconditioned with 3 mL of methanol followed by 3 mL of deionized water immediately before use. After sample loading, the cartridge was rinsed with 5 mL of deionized water, followed by elution with 6 mL of acetone. The eluted fractions were collected, evaporated to dryness, and reconstituted in 20 mL of electrolyte solution for further electrochemical analysis.

2.5. Data analysis

The method performance was evaluated based on linearity, limits of detection (LOD) and quantification (LOQ), precision, and accuracy. The LOD and LOQ were calculated using the following equations:

$$\mathrm{LOD} = \frac{3\,SD}{s}$$

$$\mathrm{LOQ} = \frac{10\,\mathrm{SD}}{s}$$

where *SD* is the standard deviation of the peak current obtained from three replicate measurements at the lowest concentration level on the calibration curve, and *s* represents the slope of the corresponding calibration equation.

3. RESULTS AND DISCUSSION

3.1. Electrochemical behavior of tetracycline at activated GCE

The voltammetric behavior of 100 μmol L^{-1} TC on the activated GCE was performed by CV measurements. 0.2 M Na_2SO_4 solution at pH 3.0 was selected as the optimized electrolyte according to previous reports with some modifications [24]. As shown in Figure 1, during the anodic scan from –0.20 V to +1.50 V, two broad oxidation peaks were observed at approximately +1.05 V and 1.18 V *vs.* Hg/Hg_2Cl_2 (3 M KCl)). No reduction peak was detected in the reverse cathodic scan, indicating an irreversible electrochemical reaction. According to the literature, the electro-oxidation of TC proceeds via a two-step mechanism involving the transfer of two protons and two electrons ($2H^+/2e^-$) (Figure 2) [25-27]. The first step involves the oxidation of the phenolic moiety at two distinct positions (ortho and para) with the addition of hydroxyl groups. In highly acidic environments, the resulting product undergoes further oxidation due to strong intramolecular

hydrogen bonding interactions [16]. For the purpose of quantification, the first oxidation peak was selected, as it exhibited enhanced sensitivity.

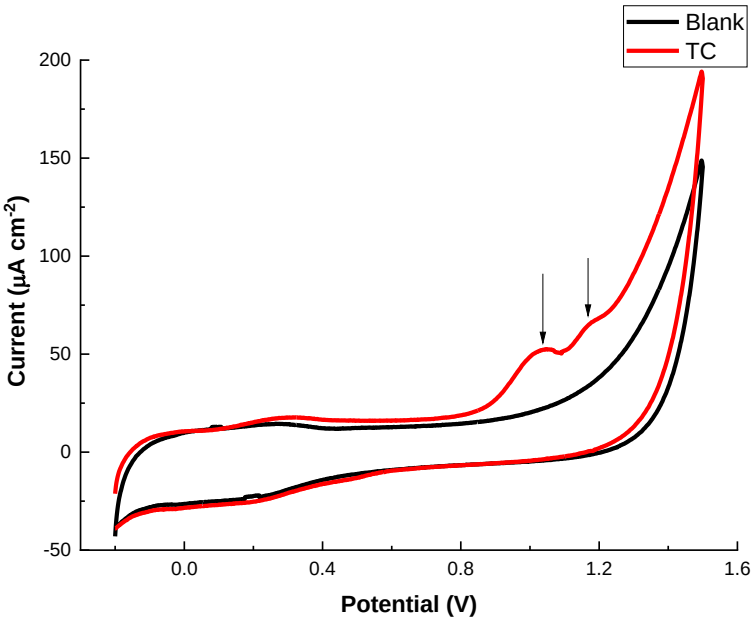

Figure 1. CV voltammogram of 100 μmol L^{-1} TC in 0.2 M Na_2SO_4 solutions at pH 3.0 has two potential peaks in +1.05V and +1.18 V

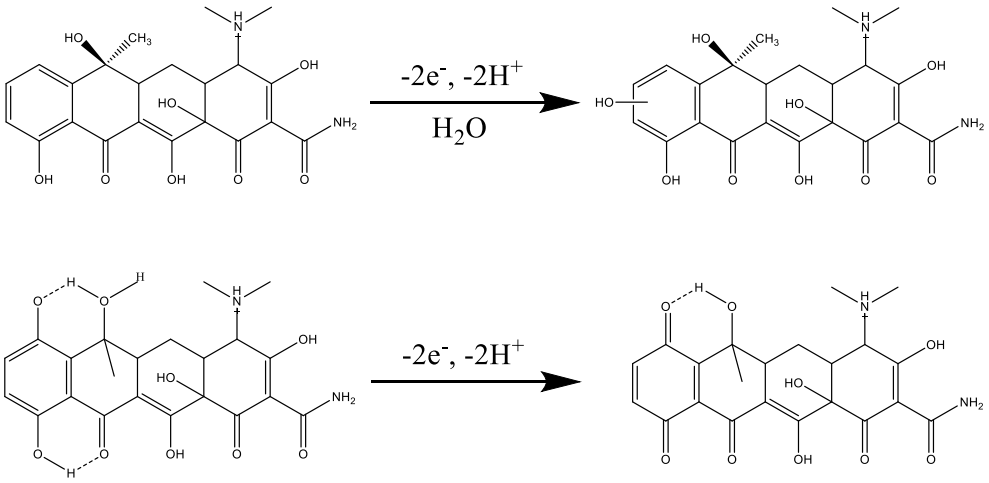

Figure 2. The electrochemical reaction mechanism of TC

3.2. The electrochemical response of tetracycline in the presence of surfactants

Surfactants, amphiphilic molecules containing hydrophobic head and hydrophilic tail group, have widely been used to increase the sensitivity of electrochemical method [28-30]. It is assumed that surfactants adsorbed on the surface of the electrode/solution can change the interface between the electrode and the solution and the electrochemical process taking place there and thus also increase the detection limit of analytes [28, 31]. For this purpose, several surfactants, including

SDS, CTAB, and Triton X-100, were investigated. Figure 3 indicates the impact of surfactant type on the anodic peak current of TC. The addition of neutral Triton X-100 causes a decrease in the oxidation current. Conversely, no significant change in the oxidation peak current was observed with the addition of CTAB. In contrast, the incorporation of SDS led to a substantial increase in the oxidation current. It should be noted that the surface of GCE is initially hydrophobic. It is important to note that TX-100 has both a hydrophobic tail and head group, while SDS and CTAB feature a long hydrophobic tail coupled with a hydrophilic head group. In this context, the hydrophobic tails of these surfactants are attached to the surface of the GCE electrode through the hydrophobic interactions. Consequently, the hydrophilic head groups of the surfactants are oriented towards the bulk solution. Therefore, adsorption on the electrode surface is likely influenced by the interactions between the negatively charged (SDS), positively charged (CTAB), or neutral (TX-100) head groups and the positively charged TC molecules at pH 3.0. The enhancement effect of SDS in the increase of the oxidation current of TC could be due to the accumulation of the amine group in TC at the negatively charged surfactant layers on electrode surfaces. Therefore, SDS was selected as the optimum surfactant for further experiments.

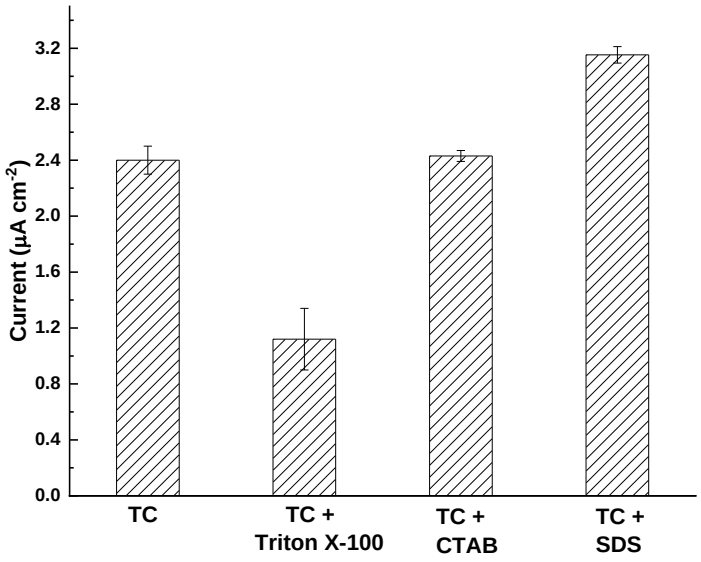

Figure 3. Effect of surfactant type on the anodic peak current of 2.0 μmol L^{-1} TC

Measurements carried out in 0.2 M Na_2SO_4 solutions (pH 3.0), [surfactant] = 1.0 μmol L^{-1}

The effect of SDS concentration was investigated within the range of 0.1 to 100 μmol L^{-1}. As illustrated in Figure 4, the oxidation peak current increased with rising SDS concentrations up to 1.0 μmol L^{-1}, indicating enhanced preconcentration of TC on the electrode surface. However, further increases in SDS concentration beyond 1.0 μmol L^{-1} resulted in a decline in the oxidation peak current, likely due to the formation of micellar structures that hinder analyte interaction with the electrode surface. Consequently, an SDS concentration of μmol L^{-1} was selected as the optimal condition for TC determination.

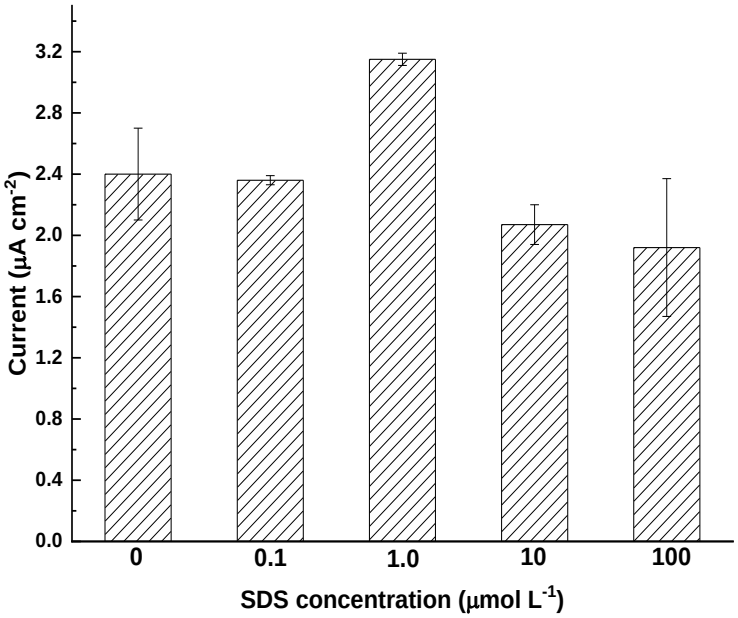

Figure 4. Influence of SDS Concentration on the anodic peak current of 2.0 μmol L^{-1} TC. Measurements carried out in 0.2 M Na_2SO_4 solutions (pH 3.0), [SDS] in the range of 0.1 to 100 μmol L^{-1}

3.3. Establishment of the Calibration Curve, LOD and LOQ

DPV technique was employed for the quantification of TC under optimized experimental conditions. Measurements were conducted using an activated GCE in 0.2 M Na_2SO_4 solution at pH 3.0, supplemented with 1.0 μmol L^{-1} SDS. The resulting differential voltammograms, shown in Figure 5A, demonstrate a progressive increase in oxidation peak currents with increasing TC concentrations. The relationship between the oxidation peak currents and TC concentrations is presented in Figure 5B, showing two distinct linear ranges: 1.0 – 10 μmol L^{-1} and 10 –100 μmol L^{-1}, at an applied potential of 0.85 V *vs.* Hg/Hg_2Cl_2 (3 M KCl). The corresponding linear regression equations were expressed as I (μA) = 0.870 C (μmol L^{-1}) + 1.048 ($R^2 = 0.990$) and I (μA) = 0.145 C (μmol L^{-1}) + 8.730 ($R^2 = 0.991$). The calculated LOD and LOQ were 0.31 μmol L^{-1} and 0.92 μmol L^{-1}, respectively. Compared to previous studies related to electroanalytical methods for TC detection, the proposed method in this study exhibited good analytical performance in terms of LOD and working range (Table 1).

The reproducibility of the activated GCE was evaluated by performing six consecutive determinations of 2.0 μmol L^{-1} TC. After each determination, the used electrode undergoes the surface treatment step to remove any adsorbents and activate the electrode surface. The measurements demonstrated good reproducibility, with a relative standard deviation (RSD) of 4.3%. The DPV response for six measurements at the same activated GCE for TC solution of 2.0 μmol L^{-1} TC with the RSD was about 3.6%, thus confirming the reusability of the method.

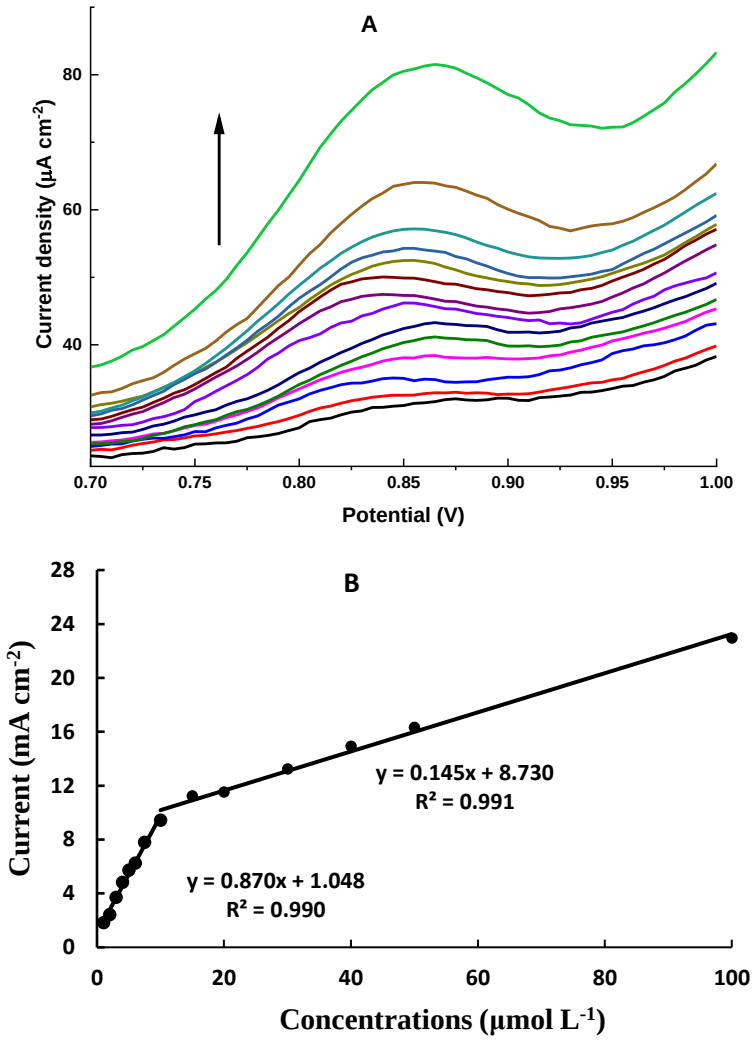

Figure 5. DPV voltammograms (A) and calibration plots (B) for TC (1.0 – 100.0 µmol L^{-1}) in 0.2 M Na_2SO_4 (pH 3.0) with 1.0 µmol L^{-1} SDS

Table 1. Evaluation of the proposed method against existing electroanalytical approaches for TC detection

Electrode	Working range (µmol L^{-1})	LOD (µmol L^{-1})	Reference
Multi-walled carbon nanotubes/carbon paste electrode	20 – 310	0.36	[32]
Molecular-imprinted polymer/screen-printed carbon electrode	0.05 - 10	0.03	[33]
Nano MnO_2/GCE	1.0 – 1000	0.51	[34]
WO_3/rGO/GCE	0.1 – 400	0.20	[35]
MnO_2@Zr-MOF/GCE	2.0 – 200	0.26	[36]
This study	1.0 – 100	0.31	

3.4. Interferences and their elimination

According to previous studies, honey contains a variety of trace elements depending on the particular botanical and geographical origin [37]. Metal ions in honey exhibit a strong propensity to interact with TC and form metal complexes [38, 39]. For example, the complex structures of Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, and Fe^{3+} with TC have been reported [40, 41]. Therefore, the determination of TC using the electrochemical method may be influenced by the presence of the metal ions.

In this study, typical metal ions including NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Al^{3+} were examined as potential interferences. The experiment was conducted using a solution containing 20 μmol L^{-1} TC and each of the interfering ions (200 μmol L^{-1}) in 0.2 M Na_2SO_4 solutions at pH 3.0, with 1.0 μmol L^{-1} SDS presence.

The results, as shown in Figure 6, indicate that the peak current of TC remained largely unaffected by the presence of interfering ions such as NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}. However, the presence of Fe^{2+}, Fe^{3+}, and Al^{3+} led to a significant change in the TC response, with a signal decrease of 70.3%, 46.2%, and 30.0% for Fe^{2+}, Fe^{3+}, and Al^{3+}, respectively. It is indicated that Fe^{2+}, Fe^{3+}, and Al^{3+} ions interfered with the determination of TC. This interference can be ascribed to the formation of complex structures of Fe^{2+}, Fe^{3+}, and Al^{3+} with TC. To eliminate their interferences, 0.5 M $H_2PO_4^-$, a relatively inactive inorganic ion, was added to the electrolyte solution to compete with TC in metal-TC complexes. As shown in Figure 7, the results indicate that the presence of $H_2PO_4^-$ ions effectively prevented Fe^{2+}, Fe^{3+}, and Al^{3+} ions from influencing the detection of TC, confirming the ability of $H_2PO_4^-$ to eliminate these interferences. Therefore, 0.5 M $H_2PO_4^-$ is proposed to minimize such interferences in TC determination.

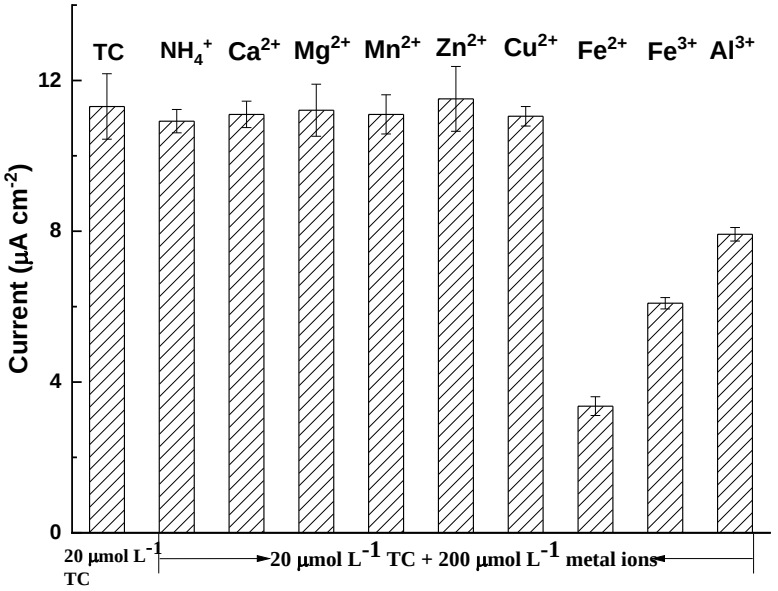

Figure 6. Effect of interference species on the detection of TC (20 μmol L^{-1}) with 10-fold of NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Al^{3+}.

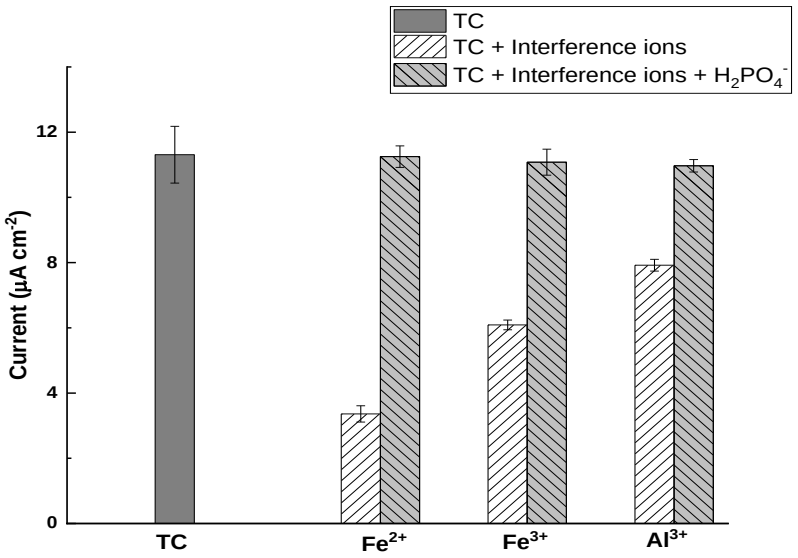

Figure 7. Elimination of interference ions (Fe^{2+}, Fe^{3+}, Al^{3+}) by $H_2PO_4^-$. Measurements carried out in 0.2 M Na_2SO_4 solutions (pH 3.0), [TC] = 20 μmol L^{-1}, [SDS] = 1.0 μmol L^{-1}, [Interference ions] = 20 μmol L^{-1}, [$H_2PO_4^-$] = 0.5 mol L^{-1}

3.5. Determination of tetracycline in honey samples

To further assess the analytical reliability and practical applicability of this electrochemical assay, it was applied to determine TC residues in honey samples using the standard addition method. To minimize matrix effects and other potential interferences, the honey samples were purified through solid-phase extraction. The recoveries were calculated using the linear calibration curves and the results are presented in Table 2. The real honey sample recovery rate ranged between 81.8% and 91.8%, and the RSD value was <5%. These findings validate that the electrochemical assay, in combination with solid-phase extraction, is a robust and reliable method for detecting TC residues in honey samples.

Table 2. Analysis results of some spike samples on Vietnamese honey

Sample	TC spiked (μmol L^{-1})	TC recovery (μmol L^{-1})	Recovery (%)
S01	5.0	4.22 ± 0.09	88.5
S02	5.0	4.09 ± 0.10	81.8
S03	5.0	4.37 ± 0.13	87.4
S04	5.0	4.59 ± 0.16	91.8
S05	5.0	4.44 ± 0.21	88.8

4. CONCLUSION

In summary, a simple and effective electrochemical method for detecting TC in honey samples using activated GCE has been developed. The addition of the surfactant SDS

significantly enhanced the peak current of TC in comparison to the unmodified bare electrode. Under the optimum conditions, TC detection was performed using DPV, which provided two linear concentration ranges of 1.0 to 10 μmol L^{-1} and 10 to 100 μmol L^{-1}, demonstrating high stability and reproducibility. The LOD and the LOQ for TC were calculated to be 0.31 μmol L^{-1} and 0.92 μmol L^{-1}, respectively. The interferences of many potential metal ions and their elimination have been illustrated. Furthermore, the combination of SPE and electrochemical method allows determination of TC residues in honey samples, with recovery rates ranging from 81.8% to 91.8%. These results suggested that this methodology could be used as a promising method in potentially practical applications.

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Declarations of interest

The authors declare no conflict of interest in this reported work.

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