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Gold-Modified Electrochemical Sensor for Rapid Detection of Muscarine in Mushroom Poisoning

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Abstract

Muscarine is a toxic alkaloid produced by neurotoxic mushrooms, known for its heat stability. Ingesting muscarine-containing mushrooms rapidly stimulates the parasympathetic nervous system within 15 min, leading to mushroom poisoning. To address this issue, we developed an electrochemical sensor using a screen-printed carbon electrode (SPCE) modified with gold to detect muscarine. The electrochemical behavior of muscarine was studied in acidic conditions using 10 mM potassium dichromate ($K_2Cr_2O_7$) as an oxidizing agent and DI water as the supporting solution. The cathodic current responded to the muscarine concentration which was detected in 2 min of reaction time by differential pulse voltammetry. Under optimal conditions, the detection limit was 4.07 µg/mL. The developed muscarine electrochemical sensor was tested on mushroom samples, and the results were compared with chemical and morphological identification. The gold-modified SPCE, combined with dichromate ($Cr_2O_7^{2-}$) as a reagent, showed promise for development as a point-of-care testing electrochemical sensor capable of detecting muscarine in mushroom samples.

Keywords: Muscarine, Electrochemical sensor, Gold-modified SPCE, Potassium dichromate, Neurotoxic mushrooms

Introduction

Poisonous mushrooms are a public health concern due to their toxic effects, especially on the nervous system, and symptoms appear rapidly. Muscarine mimics the action of acetylcholine by binding to acetylcholine receptors, leading to overstimulation of the parasympathetic nervous system, with sweating, tearing, and excessive salivation occurring within 15 min [1].

Methods used to determine muscarine include chemical assays for screening and confirmation, and morphological identification combined with DNA barcoding to identify mushroom species that contain muscarine [1,2], with thin layer chromatography (TLC) [3] and liquid chromatography-mass spectrometry (LC-MS) [4] common chemical diagnostic mechanisms. Muscarine has been analyzed by many LC-MS techniques such as electrospray liquid chromatographic-mass spectrometry (LC/ESI-MS) [5], liquid chromatography-time-of-flight mass spectrometry (LC-TOF MS) [6,7], ultrahigh-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [8], and capillary electrophoresis coupled with electrospray tandem mass spectrometry (CE–ESI-MS/MS) [9]. These methods provide precise results and low detection limits but rely on specialized equipment, trained personnel, and extensive resources. Limitations include high cost, long analysis time, and large amounts of chemicals and samples. Moreover, these methods cannot be used for field measurements or point-of-care diagnostics. The development of electrochemical sensors for muscarine detection has emerged as a promising alternative but has not been extensively reported. Electrochemical sensors offer advantages including portability, cost-effectiveness, high sensitivity, low detection limit, stability and selectivity. Biomolecules, organic substances, and nanomaterials can be used to interact with or modify toxins to enhance sensing performance. Aptamers, nucleic acid or single-stranded DNA are particularly useful for the detection of toxins such as Staphylococcal enterotoxin B (SEB) [10], Fumonisin B1 (FB-1) [11], and alpha-amanitin [12,13]. Nanoparticles offer advantages in directly bonding or interacting with biomolecules including stabilizing modifications on sensors, as well as their high electroconductivity and sensitivity. Sensors have also been developed with silver nanoparticles (AgNPs), graphene oxide (GO) [14], poly-o-aminophenol-carbon nanotubes (PoAP-CNTs) [15], and streptavidin-biotinylated bacteriophages [16]. Gold composites are highly effective for modification on electrodes or sensors, improving electrical conductivity, biocompatibility, and the ability to combine with other materials such as gold nanoparticles (AuNPs) [17-20], reduced graphene oxide/gold nanoparticles/4-mercaptophenylboronic acid (rGO/AuNPs/MPBA) [21], AuNPs modified on (3-mercaptopropyl) trimethoxysilane (MPTS) [22], and AuNPs deposited on the surface of graphene oxide layers (Au/GO) [23]. Biomolecules, organic substances, and nanomaterials positively impact the development of sensors, enhancing efficiency in measuring toxic substances and reducing interference from other matrices in the samples.

Electrochemical analysis is an interesting approach to develop sensors for determining muscarine in toxic mushrooms in food poisoning situations. Gold (Au) was attached to a screen-printed carbon electrode (SPCE) to increase the efficiency of measuring muscarine toxins using $Cr_2O_7^{2-}$ as an oxidizing agent. This electrochemical analysis method presents a new protocol for muscarine detection. The sensor analyzed biological toxins with high sensitivity, low cost, low reagent consumption and rapid analysis, and showed promise for toxin analysis in the field.

Materials and methods

Chemicals and materials

Standard muscarine, gold(III) chloride trihydrate ($HAuCl_4.3H_2O$), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$), nickel hydroxide ($Ni(OH)_2$), and nickel oxide (NiO) were purchased from Sigma-Aldrich. Hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Merck. A 10 mM ferri/ferro cyanide solution ($[Fe(CN)_6]^{3-/4-}$) was prepared by mixing Fe^{3+} and Fe^{2+} solution in a 1:1 ratio. $K_2Cr_2O_7$ was dissolved in water (HPLC grade, Labscan) and acidified with concentrated sulfuric acid (H_2SO_4, J.T.Baker) to prepare a dichromate solution. The SPCE was obtained from Zensor.

Characterization of muscarine reaction and SPCE modification

Muscarine electrochemical behavior was studied using reagents, acid and base including $Cr_2O_7^{2-}$, $Ni(OH)_2$, HCl, and NaOH in a concentration of 10 mM, with 25 µL of muscarine solution mixed with 25 µL of the reagent on the SPCE surface. The reactions were characterized using cyclic voltammetry (CV) within the range of −1.0 to +1.0 V at a scan rate of 0.05 V/s (EmStat4s, PalmSens).

The SPCEs were modified with silver nanoparticles (AgNPs) [13], graphene oxide (GO) [13], gold (Au) and NiO. For AgNPs, GO, and NiO, 50 µL of a 2 mg/mL solution was dropped onto the SPCE surface and dried at room temperature. By contrast, Au (2 mg/mL, 50 µL) was electrodeposited onto the SPCE using chronoamperometry at −1.25 V (vs Ag/AgCl) for 300 s to form an Au layer [18]. The modifications on the SPCE were characterized in 10 mM $[Fe(CN)_6]^{3-/4-}$ solution using cyclic voltammetry (CV) within the range of −1.0 to +1.0 V at a scan rate of 0.05 V/s. Scanning electron microscopy (SEM) (TESCAN, VEGA4) and energy-dispersive spectroscopy (EDS) were used to examine the surface morphology and confirm the material elements of both bare and modified SPCEs.

For conducting high sensitivity and high electrode surface area, the amounts of Au on SPCE were varied between 4 and 40 µg. Different volumes of 2 mg/mL gold solution were dropped onto the surface of the SPCE and electrodeposited at −1.25 V (vs Ag/AgCl) with a scan rate of 0.05 V/s using chronoamperometry for 300 s. The response signal was measured in 10 mM $[Fe(CN)_6]^{3-/4-}$ solution using differential pulse voltammetry (DPV) ranging from −1.0 to +1.0 V with a 0.05 V/s scan rate.

Electrochemical measurement

The SPCE was modified with 50 µL of gold solution (2 mg/mL), washed with water and dried at room temperature. Then, 25 µL of $Cr_2O_7^{2-}$ solution was used to oxidize the hydroxyl group of muscarine to the carbonyl group by dropping it onto the SPCE/Au surface, followed by the addition of muscarine solution (25 µL). The mixed solution was analyzed by DPV in the range +1.0 to −1.0 V at a scan rate of 0.05 V/s. The current signal corresponding to $Cr_2O_7^{2-}$ was measured and calculated using the differential cathodic peak current at +0.100 V (vs Ag/AgCl) ($\Delta I = I_0 - I$) between the blank (I_0) and after adding the muscarine (I). The protocol is shown in **Figure 1**.

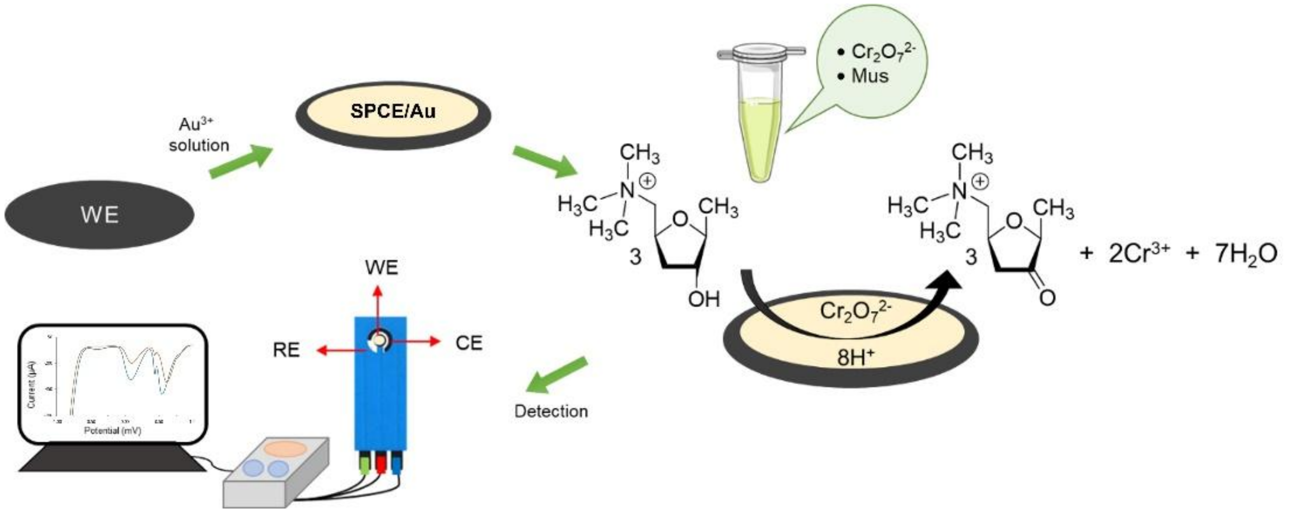


Figure 1 Schematic representation of the electrochemical determination of muscarine (WE: Working electrode, RE: Reference electrode and CE: Counter electrode).

Performance of the sensor

The sensor was used to determine muscarine following the method described in the electrochemical measurement section under optimal conditions. The differential cathodic peak current was +0.100 V (vs Ag/AgCl). Linearity was detected at muscarine concentrations of 4.07 to 40.70 µg/mL by DPV. The limit of detection (LOD) was determined by measuring the current at the lowest concentration of muscarine with 3 repetitions. Seven electrodes were tested for reproducibility and measured muscarine at 32.56 µg/mL. The selectivity of the sensor was tested with methanol, ethanol, and iso-propanol at a concentration of 32.56 µg/mL.

Sample preparation

The developed sensor was used to measure muscarine content in 10 mushroom samples collected from mushroom poisoning cases in Thailand, with the analysis confirmed by the Toxicology Center, National Institute of Health of Thailand (Department of Medical Sciences, Ministry of Public Health). A 20 mg amount of each dried sample was cut into small pieces and 500 µL of DI water was added. The sample was mixed well and soaked for 30 min. The extracted solution was then determined for muscarine following the developed method.

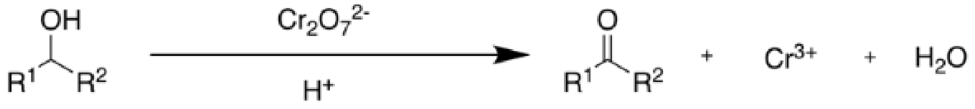
Chemical and morphological identification

Ten samples of toxic mushrooms including both muscarine-producing and non-muscarine-producing varieties, as well as food contaminated with muscarine, were obtained from poisoning cases and used in this study. Chemical and morphological identification were conducted based on Parnmen *et al.* [1].

Results and discussion

Characterization of muscarine reaction

Muscarine contains the secondary alcohol functional hydroxy group and the commonly used $Cr_2O_7^{2-}$ oxidizing agent was investigated in the oxidation reaction before testing with $Ni(OH)_2$, HCl, and NaOH, and further measurements using CV. Results showed that the current signal of muscarine with NaOH and $Ni(OH)_2$ was at the baseline, while $Cr_2O_7^{2-}$ and HCl produced measurable signals, as shown in **Figure 2(A)**. The anodic and cathodic peak currents of the muscarine reaction with $Cr_2O_7^{2-}$ were observed at +0.141 and −0.029 V, respectively.



In this reaction, the hydroxyl group is oxidized to a carbonyl group by $Cr_2O_7^{2-}$. The hydroxyl group of muscarine reacted in a similar manner, leading to electron transfer to the electrode and detection using electrochemical techniques. Moreover, $Cr_2O_7^{2-}$ provided a more pronounced current signal compared to the others and was used to study the reaction of muscarine.

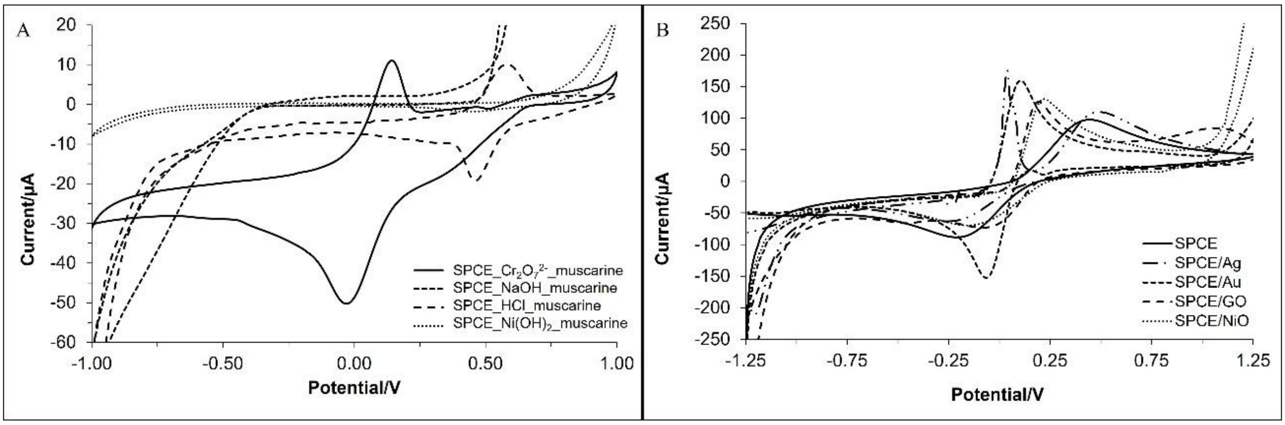


Figure 2 (A) Cyclic voltammogram of muscarine reaction with $Cr_2O_7^{2-}$, NaOH, HCl and $Ni(OH)_2$ with a scan rate of 0.05 V/s. (B) Cyclic voltammogram of SPCE, SPCE/AgNPs, SPCE/ Au, SPCE/GO and SPCE/NiO in 0.01 M $[Fe(CN)_6]^{3-/4-}$ solution with a scan rate of 0.05 V/s.

Modification of the SPCEs

The SPCEs were modified with AgNPs, GO, Au, and NiO to improve the electrical conductivity and electrode surface area. The SPCE and modified SPCEs were measured in a $[Fe(CN)_6]^{3-/4-}$ solution. Results in **Figure 2(B)** indicated that the Au modified SPCE (SPCE/Au) exhibited the highest current signal, with facilitated electron transfer, enhanced electrical conductivity, and high surface area [18,19]. Therefore, SPCE/Au was used as the working electrode (WE) to study muscarine reactions.

The structures of the bare and modified electrodes were examined for morphology using SEM. The bare SPCE (**Figure 3(A)**) displayed a rounded sheet-like pattern, while the SPCE/Au (**Figure 3(B)**) showed small pellets spread throughout the sheet, indicating electrodeposited Au [19]. When the SPCE/Au was modified with muscarine, clusters of the substance appeared, as shown in **Figure 3(C)**. The electrodes confirmed the material elements using EDS, as shown in **Figures 3(D) - 3(F)**. The SPCE/Au image showed that the gold element appeared on the SPCE surface. When the electrode was modified with muscarine, the nitrogen element was presented. The SEM and EDS images confirmed the successful deposition and modification of substances on SPCE.

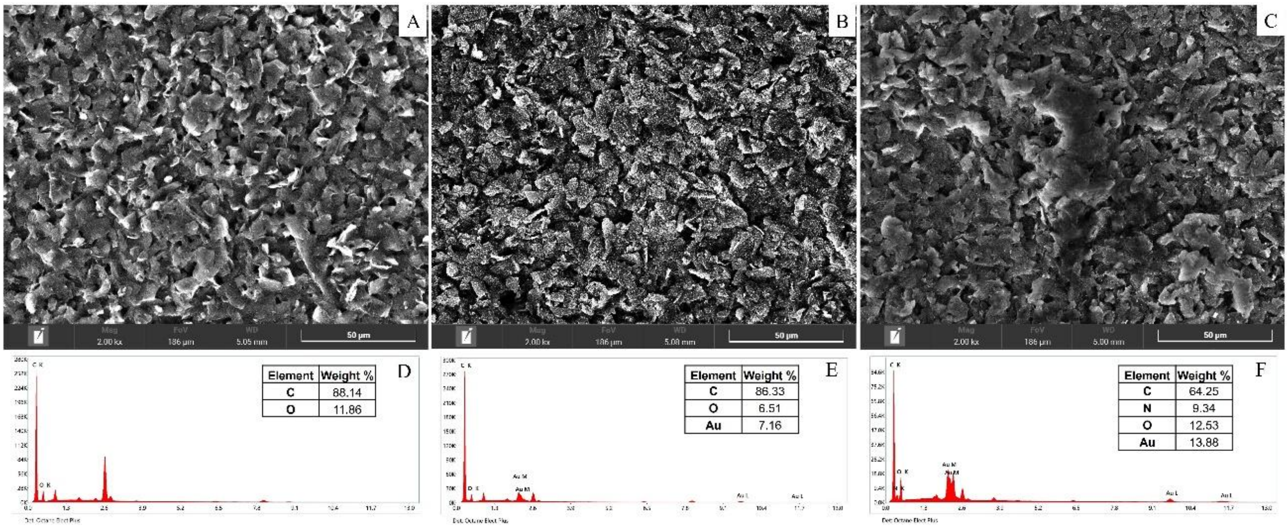


Figure 3 SEM images of SPCE (A), SPCE/Au (B), SPCE/Au/muscarine (C) and EDS images of SPCE (D), SPCE/Au (E), and SPCE/Au/muscarine (F).

Optimization of the reaction

A 2 mg/mL gold solution was electrodeposited onto the surface of the SPCE in amounts ranging from 4 to 40 µg. The DPV current in 10 mM $[Fe(CN)_6]^{3-/4-}$ solution, as shown in **Figure 4**, displayed a higher current with the addition of Au, with 30 µg of Au providing the highest current and decreasing above this concentration [17]. Therefore, 30 µg of Au was selected for further experiments.

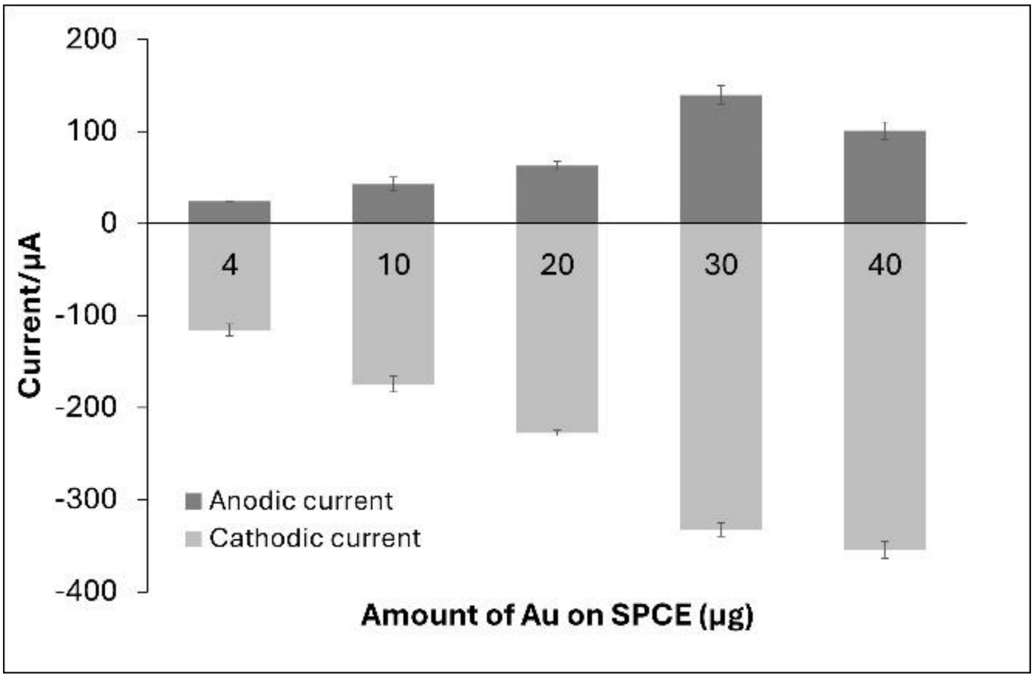


Figure 4 Relationship between the amount of Au on SPCE and current signal.

The reaction between the hydroxyl group of muscarine and the $Cr_2O_7^{2-}$ solution using SPCE/Au was characterized. Results showed that muscarine and $Cr_2O_7^{2-}$ reacted within a range of +1.0 to −1.0 V (**Figure 5(A)**). The cathodic peak current of $Cr_2O_7^{2-}$ on bare SPCE was detected at 0.089 V, while the cathodic peak current of SPCE/Au was 0.089 V, indicating an increased current because of the high conductivity of Au. After $Cr_2O_7^{2-}$ reacted with muscarine, the cathodic peak current shifted to 0.100 V. This study focused on detecting the current signal during the reduction reaction, specifically the reduction of $Cr_2O_7^{2-}$.

The oxidation reaction of muscarine with $Cr_2O_7^2$ occurred under acid conditions (in H_2SO_4). Supporting solutions such as DI water, phosphate buffer (pH5), and acetate buffer (pH5) were studied, as illustrated in **Figure 5(B)**. The oxidation reaction of muscarine obtained a high delta reductive current of Cr(VI). When the phosphate buffer and acetate buffer were used in the reaction, the delta current decreased to low and moderate current levels (9.87 and 82.34 µA), respectively. These results suggested that the buffer decreased the rate of the oxidation reaction by neutralizing the concentration of acid under buffer conditions. The negative ions in the buffer solution decreased the rate of the oxidation reaction through the negative salt effect [24,25].

To optimize $Cr_2O_7^{2-}$ concentration, a solution of $Cr_2O_7^{2-}$ at concentrations of 5, 10, 25, and 50 mM in the presence of H_2SO_4 were tested, as shown in **Figure 5(C)**. Lower concentrations resulted in lower currents due to the smaller amount of reactant, while higher concentrations produced higher currents. However, at 25 and 50 mM $Cr_2O_7^{2-}$, the current decreased attributed to the wider diffusion layer blocking electron transfer on the electrode surface. Therefore, 10 mM $Cr_2O_7^{2-}$ was chosen for further experiments.

The reaction between the hydroxyl group of muscarine and $Cr_2O_7^{2-}$ solution under acidic conditions was monitored over 0 to 10 min. At 0 min, the reaction had not yet reached equilibrium. Equilibrium was established between 2 and 10 min, providing a high current. **Figure 5(D)** shows that the current was highest at 2 min, and this reaction time was used in subsequent measurements.

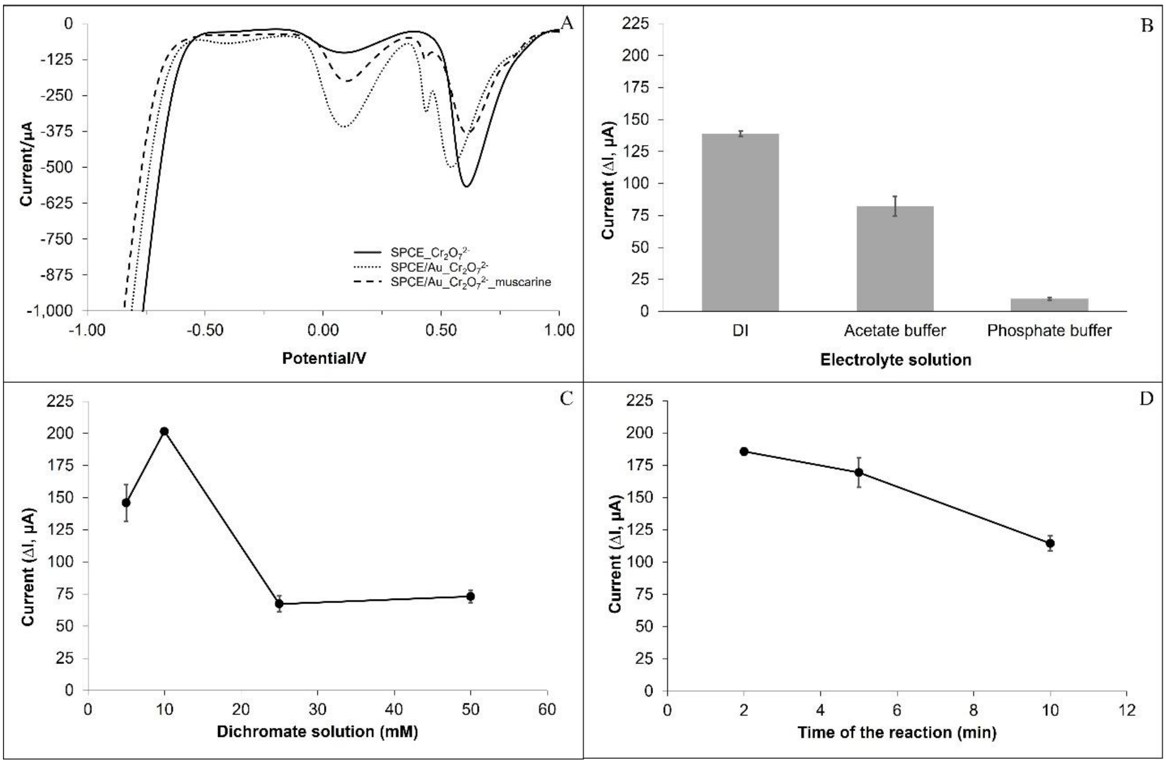


Figure 5 Differential pulse voltammogram of SPCE, SPCE/Au and SPCE/Au/muscarine in dichromate solution with a scan rate of 0.05 V/s (A) and the effects of electrolyte solution (B), concentration of dichromate (C), and reaction time (D).

Sensor performance

The sensor was used to determine muscarine that was oxidized with $Cr_2O_7^{2-}$. When the muscarine concentration increased, the cathodic current signal of $Cr_2O_7^{2-}$ decreased, while the blank correlation signal increased. Under optimal conditions, the calibration graph detected muscarine at concentrations ranging from 4.07 to 40.70 mg/L, as shown in **Figure 6**. The limit of detection (LOD) was 4.07 mg/L. The reproducibility of the sensor was assessed using 7 SPCE/Au electrodes with measurement of 32.56 mg/L of muscarine by DPV. The percentage relative standard deviation (% RSD) of the reproducibility was 3.98 %.

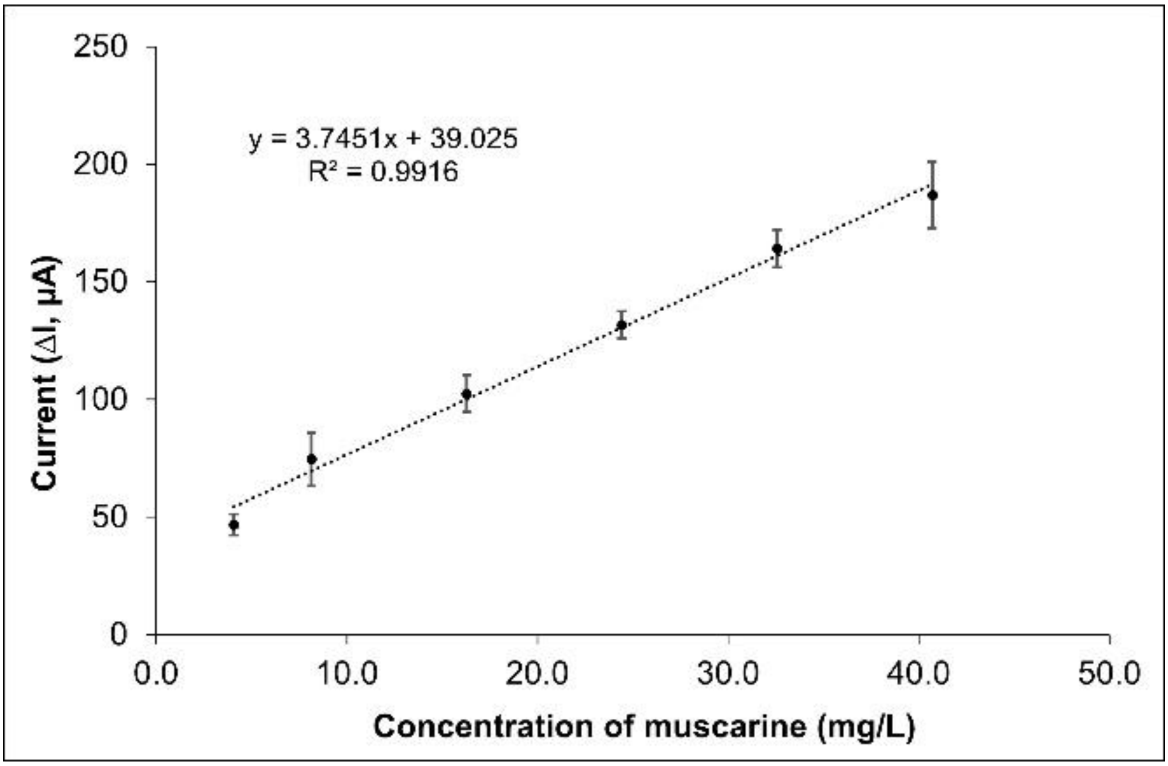


Figure 6 Relationship between the concentration of muscarine and the current signal.

The selectivity of the sensor was tested with methanol, ethanol, and iso-propanol. Results showed that the sensor detected muscarine, methanol, ethanol, and iso-propanol at 0.100, 0.049, 0.049, and 0.149 V, respectively. Therefore, muscarine detection was selective at 0.100 V.

The developed sensor was used to study muscarine in mushroom samples extracted with DI water. $Cr_2O_7^{2-}$ and the sample solutions were applied to the SPCE surface, and muscarine was determined using the DPV technique under the studied conditions, with results shown in **Table 1**.

Table 1 Determination of muscarine in mushroom samples.

Sample no.	Morphological identification combined with DNA barcoding	LC-MS/MS	Developed sensor
1	*Inosperma*	+	+
2	*Inosperma*	+	+
3	*Pseudosperma*	+	+
4	*Pseudosperma*	+	+
5	*Inosperma*	+	+

Sample no.	Morphological identification combined with DNA barcoding	LC-MS/MS	Developed sensor
6	*Inosperma*	+	+
7	Food with mushrooms	+	ND
8	Food with mushrooms	+	ND
9	*Inocybe*	-	-
10	*Inocybe*	-	-

[+] positive. [-] negative. [ND] non detected.

Qualitative testing using liquid chromatography-mass spectrometry (LC-MS/MS) and the developed sensor provided consistent results. The LC-MS/MS determination exhibited muscarine with ionic transitions of precursor ion (m/z 174) and product ion (m/z 57) [1,5,9], as shown in **Figure 7**. However, detecting muscarine in mushroom-containing foods was difficult due to the matrix effect on the food samples. Studying the matrix effect and developing reliable extraction methods represent challenges for future research.

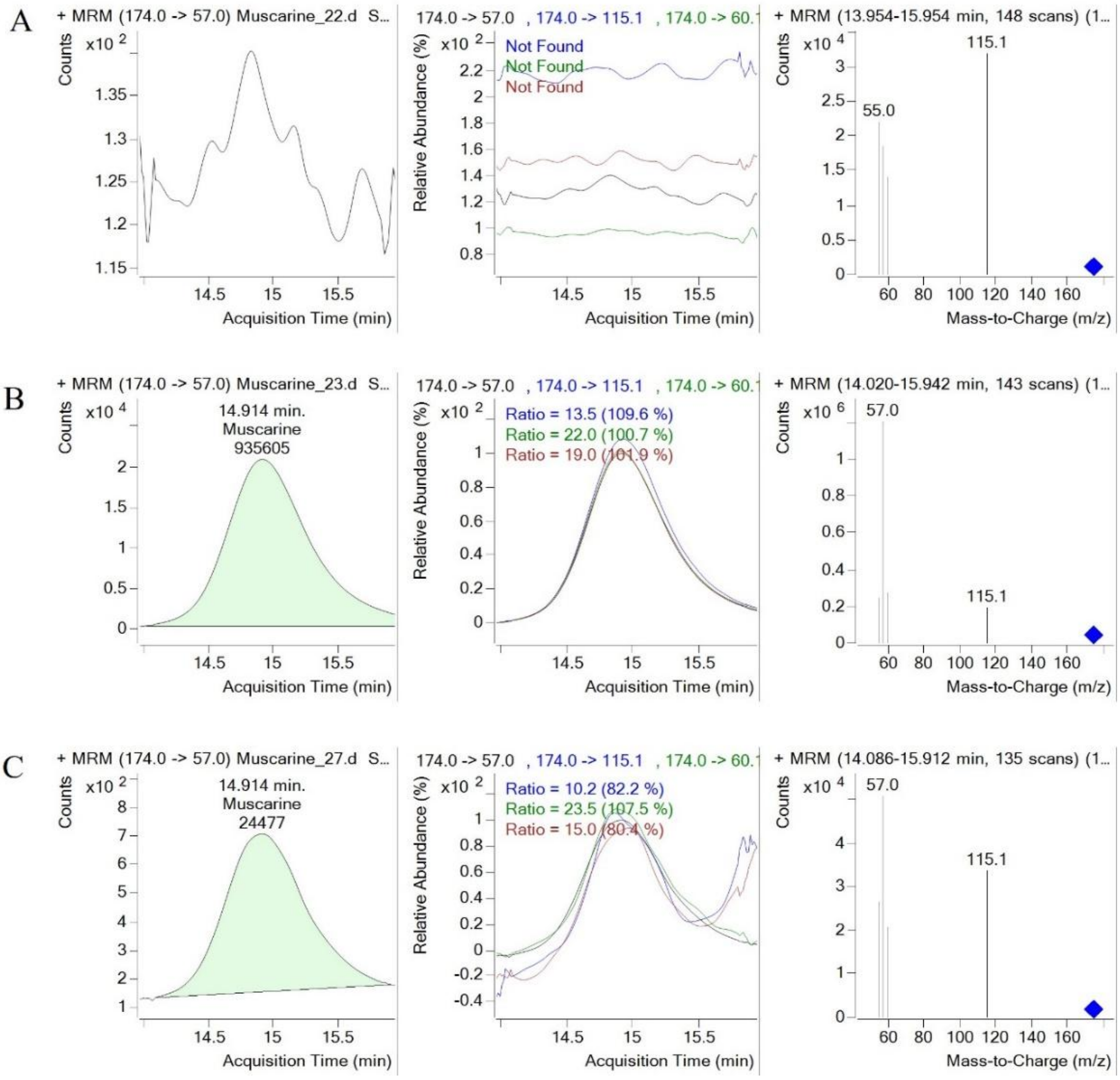


Figure 7 Liquid chromatography-tandem mass spectrometric MRM chromatograms of blank: acetonitrile (A), standard muscarine (B), and mushroom sample (C) with ionic transitions of precursor ion (m/z 174) and product ion (m/z 57).

Conclusions

This study highlighted the development of an electrochemical sensor for detecting muscarine in mushrooms based on its reaction with the oxidizing substance, $Cr_2O_7^{2-}$, and subsequent measurement of the current response of $Cr_2O_7^{2-}$ reduction on SPCE/Au. Under the studied conditions, muscarine was detected with LOD of 4.07 mg/L. Qualitative testing showed that the results were 80 % consistent with LC-MS/MS. However, the developed sensor is not as sensitive as the LC-MS technique [5-9] but can enhance the potential for laboratory analysis, with possible adaptation for use in point-of-care testing in the field. Moreover, the matrix effect from muscarine food contamination has a challenge to develop in the future.

Acknowledgements

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