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# A label-free impedimetric immunosensor for detection of 1-aminohydantoin residue in food samples based on sol–gel embedding antibody

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ABSTRACT

A novel label-free impedimetric immunosensor for detection 1-aminohydantoin (AHD) was first constructed by the silica sol–gel immobilization of monoclonal antibody against 1-aminohydantoin antibody (AHD-McAb) on the surface of glassy carbon electrode (GCE). The electrochemical impedance spectroscopy of ferricyanide was used as a marker to probe the interface and as a redox probe to determinate AHD. The effect of operational parameters, such as amount of immobilized AHD-McAb, pH, incubation time, and incubation temperature, has been explored for the optimum analytical performance of the impedimetric immunosensor. Under the optimized conditions, the change in impedance was proportional to AHD concentrations in the range of 2.0–1.0 × $10^3$ ng/mL ($r = 0.9990$) with the detection limit of 2.0 ng/mL. The specificity, reproducibility, stability, and accuracy of the proposed impedimetric immunosensor were also evaluated. In addition, the proposed immunosensor was successfully applied for the determination of AHD in food samples using the standard adding method with recoveries of 93.7–104.9%. The results obtained by the proposed immunosensor corroborate very well with the method of HPLC–MS/MS for the determination of AHD in food samples.

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## 1. Introduction

Nitrofurans are a group of synthetic broad-spectrum antibacterial drugs with a characteristic 5-nitrofuran ring group. They are widely used for the treatment of gastrointestinal and dermatological infections in animal husbandry and as growth promoters (Cooper, Samsonova, Plumpton, Elliott, & Kennedy, 2007; Szilard & Calle, 2006). Due to the potentially carcinogenic and mutagenic effects of their metabolites, the use of nitrofurans has been banned within the countries of the European Union for about 20 years. In order to protect human health, the other countries, such as China, United States, and so on, have established relevant system to ban the use of nitrofurans in aquaculture and animal husbandry. Nitrofurantoin, which belongs to the group of nitrofuran antibacterial agents, has still been used due to its low cost and high benefit till now. Its metabolite, 1-aminohydantoin (AHD), is regarded as the marker residue of nitrofurantoin for monitoring purposes. In order to detect AHD residue in food samples, the different strategies, such as enzyme-linked immunosorbent assay (ELISA) (Jiang et al., 2012), and HPLC–MS/MS (Bock, Gowik, & Stachel, 2007; Finzi, Donato, Sucupira, & Nucci, 2005; Leitner, Zollner, & Lindner, 2001; Verdon, Couedor, & Sanders, 2007), have been employed for the detection of AHD level. In the above mentioned methods, the great efficient methodology of liquid chromatography tandem mass spectrometry (LC–MS/MS) has been applied to detect the AHD in edible tissues, and it can reach the residue criterion of the minimum required performance limit (MRPL) for 1 μg/kg established by the EU Commission Decision. However, HPLC–MS/MS methodology for the detection of AHD residues involves tedious extraction, clean-up steps and derivation prior to chromatography, which is time-consuming and expensive. Thus, a sensitive and simple AHD detection method is greatly needed.

Biosensor technology is a promising analytical method for the determination of drug residue in food samples. Out of the many biosensors that exist, the label-free impedimetric immunosensor, which eliminates the labelling and catalyzing process of enzyme, is a highly sensitive and quick responsive detection of drug residues in food samples (Ionescu et al., 2007; Thavarungkul, Dawan, Kanatharana, & Asawatreratanaku, 2007).

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Sol–gel-derived silica-based material has been proved to perform a number of advantages, such as low-temperature encapsulation of biorecognition elements, tunability of physical characteristics, tunable porosity, high thermal stability, chemical inertness, experience negligible swelling in aqueous solution, and low chemical reactivity (Joesph & Prasad, 1998; Wang et al., 1999), which make it of extreme potential in the preparation of electrochemical sensors (Joesph & Prasad, 1998; Li, Chia, Goh, & Ta, 1998; Li, Tan, & Ge, 1996; Nadzhafova, Etienne, & Walcarius, 2007; Raghu, Swamy, Reddy, Chandrashekar, & Reddaiah, 2012; Wang et al., 1999).

In our research group, we tried to fabricate the electrochemical immunosensors by immobilizing different antibodies with different materials and carry out the detection of different nitrofurans' residues, respectively. In our early work, three novel label-free electrochemical impedimetric immunosensors for sensitive detection of metabolite of furaltadone, 5-morpholino-3-amino-2-oxazolidone (AMOZ), 3-amino-2-oxazolidone (AOZ), and semicarbazide (SEM), were fabricated (Jin et al., 2011; Jin, Yang, Shao, & Qin, 2013; Yang et al., 2011). To the best of our knowledge, relatively little research has been reported for detection of 1-aminohydantoin (AHD) based on label-free impedimetric immunosensor. This paper describes the preparation and characterization of sol–gel-derived thick-film impedimetric immunosensor for the detection of AHD. The sensitive detection of AHD was demonstrated based on the change in impedance before and after the antigen–antibody reaction. This immunosensing method is simple and easy, which may provide the potential application for the ultrasensitive detection of AHD residue. The experimental results showed that the detection ability of the proposed method was compared with that of LC–MS/MS.

## 2. Experimental

### 2.1. Chemicals and reagent

1-Aminohydantoin (denoted as AHD, >99%) was purchased from Shanghai Quandao Company. 4.0 mg/mL monoclonal antibody against AHD (denoted as AHD-McAb) was made by College of Veterinary Medicine, Yangzhou University. Triton X-100 and Tetraethylorthosilicate (TEOS, 99%) were purchased from Shanghai Reagent Company. All of the other chemical reagents used were analytical grade without further purification. All solutions were made up with doubly-distilled water of 18 MΩ purified from a Milli-Q purification system.

### 2.2. Apparatus

The experiments of electrochemical impedance spectroscopy (EIS) were performed with an Autolab Electrochemical Analyzer (Ecochemie, Netherlands) coupled to a one-compartment three-electrode cell. A Randles equivalent circuit was used to fit the obtained impedance spectra using the software which is equipped by instrument of an Autolab Electrochemical Analyzer. The three-electrode system consists of a chemically modified glassy carbon electrode as working electrode, a saturated calomel reference electrode (SCE) as reference electrode, and a platinum wire electrode as counter electrode. The Nyquist plots were recorded in a frequency range of $10^{-1}$–$10^{6}$ Hz. All tests were conducted on an open circuit potential, and a single modulated AC potential of 10 mV was applied for impedance measurement. All measurements were performed in the presence of a 1.0 mmol/L $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ (1:1 mixture) as a redox probe in 0.1 mol/L phosphate buffer solution (PBS, pH 7.5) and 0.1 mol/L $KNO_3$ at 25 ± 0.5 °C. S-480II FESEM scanning electron microscope (Hitachi, Japan) was used for surface image measurements.

### 2.3. Preparation of silica sol–gel solution

The silica sol–gel solution was prepared based on the previous report (Liang, Qiu, & Cai, 2005). Briefly, homogeneous silica sol–gel was prepared by mixing 50 μL TEOS, 160 μL water, 30 μL ethanol, 10 μL 0.1 mol/L HCl, and 20 μL Triton X-100 in a small plastic tube at room temperature. The mixture was sonicated for approximately 60 min until a clear and homogeneous solution resulted. The catalyst selected was 0.1 mol/L HCl, and the addition of Triton X-100 can prevent silica film from cracking. Then, the sol–gel was stored at room temperature for 2–3 h. The pH value of sol–gel was adjusted to 7.4 with 5 mol/L NaOH before it was used.

### 2.4. Fabrication of immunosensor and measurement procedure

The fabrication of impedimetric immunosensor and immunochemical assay format is illustrated in Scheme 1A. First, the glassy carbon electrode (GCE, 3 mm in diameter) was polished carefully with 1.0, 0.3 and 0.05 μm alumina slurry, rinsed successively with 1:1 nitric acid, acetone and doubly-distilled water. After pretreating, the glassy carbon electrode was cycled for 20 cycles in 0.5 mol/L $H_2SO_4$ with the potential range from −0.5 to +1.4 V at scan rate of 100 mV/s until the reproducible background was obtained. Then it was allowed to dry at room temperature. To immobilize AHD-McAb in the silica network, 3 μL of the AHD-McAb (4.0 mg/mL) was mixed with 100 μL silica sol–gel to form homogeneous solution. Then 20 μL solution was dropped on the surface of pretreated GCE and allowed to dry for the formation of gel under ambient condition for 24 h. After the modified electrode was rinsed with doubly-distilled water, the immunosensor (denoted as sol–gel-AHD-McAb/GCE) was obtained, which can be characterized by scanning electron micrograph (Scheme 1B (a)). The immunosensor was rinsed thoroughly in the PBS (pH 7. 5) and stored in the PBS (pH 7.5) prior to the EIS measurement.

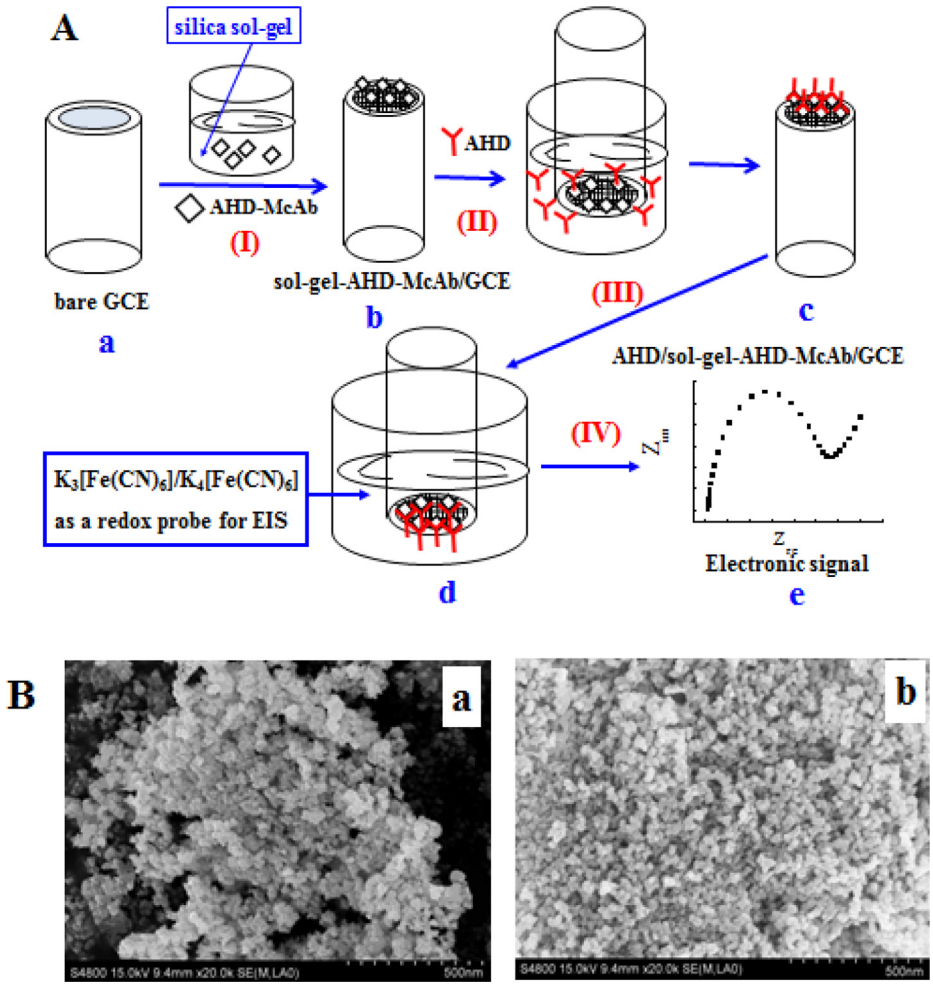
Before each measurement, the antibody-coupled electrode was incubated with different concentrations of AHD in 0.1 mol/L PBS (pH 7.5) and 0.1 mol/L $KNO_3$ for 160 min at 37 °C, the surface morphology significantly changed (Scheme 1B (b)) compared to that of sol–gel-AHD-McAb/GCE, suggesting that ADH and ADH-McAb were successfully combined on the surface of electrode. Then the resulting electrode was rinsed with doubly-distilled water, 0.1 mol/L PBS (pH 7.5) and 0.1 mol/L $KNO_3$, respectively. EIS measurements were performed in 0.1 mol/L PBS (pH 7.5) and 0.1 mol/L $KNO_3$ containing 1.0 mmol/L $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ (1:1 mixture) as a redox probe with the AC impedance method, and each impedance measurement was determined for five times.

### 2.5. Food samples

Six samples, such as pork floss, shrimp, honey, pig bladder, salted hog casings, and crab, were obtained from supermarket in Nanjing. 200 g samples of them except honey were thawed and minced in a blender, then subsampled (20 g) and stored at −20 °C in airtight containers until analysis, respectively.

### 2.6. Preparation of samples

The method of preparation sample was adopted according to previous reports (Jin et al., 2011; Yang et al., 2011). Briefly, A 2.0 g sample was weighed in a 50 mL polypropylene tube. Then 7.5 mL of water and 0.5 mL 1 mol/L HCl were added and vortex mixed for 30 s. Then pH was adjusted to 7.5 with 1 mol/L $K_2HPO_4$. After the mixture was allowed to stand for 20 min, 10 mL of ethyl acetate was added before stirring for 20 min in a rotary shaker. Then, the mixture was transferred into a polypropylene tube and centrifuged




**Scheme 1.** (A) Schematic illustration of the silica sol–gel-derived electrochemical immunosensor (I) and the working principle of the immunosensor assay (II–IV). (I) Dropped the solution of AHD-McAb in silica sol–gel on the surface of GCE, (II) the specific binding of the immobilized AHD-McAb in the competitive setup by free AHD in solution, (III) electrode was washed and transferred into the cell containing 1.0 mmol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$, (IV) EIS detection. (B) The scanning electron micrographic images of (a) sol–gel-AHD-McAb/GCE, and (b) AHD/sol–gel-AHD-McAb/GCE.

for 10 min at 4000 rpm. The ethyl acetate fractions were collected and dried at 40 °C under nitrogen. The residue was reconstituted by 2 mL of 1:1 (v/v) mixture of methanol and 0.1 mol/L PBS (pH 7.5). The buffer phase was separated by centrifugation at 4000 rpm for 10 min and collected for electrochemical immunoassay.

### 2.7. HPLC–MS/MS analysis

The conditions of the HPLC–MS/MS method were set up according to the previous report (Mottier et al., 2005). Briefly, An HPLC system with $C_{18}$ SymmetryShield LC column (15 cm × 2.1 mm i.d., 3.5 μm particle size) fitted with a SymmetryShield RP18 precolumn (1 cm × 2.1 mm i.d., 3.5 μm particle size) (Waters, Milford, MA, USA) was equilibrated with mobile phase consisting solvent A (water containing acetic acid 0.025%, v/v) and solvent B (acetonitrile) by gradient elution at a flow rate of 0.3 mL/min. The source block temperature was set at 350 °C. The dwell time for each transition reaction was set at 25 ms. The electrospray voltage was set to 5.5 kV and the collision energy to 18 eV. Nitrogen was used as collision gas.

The sample preparation of HPLC–MS/MS was pretreated according to the previous report (Mottier et al., 2005). Briefly, a well homogenized sample (2.0 ± 0.05 g) was weighed into a 50-mL Falcon polypropylene tube. 4 mL water, 0.5 mL hydrochloric acid (1 mol/L) and 150 μL O-nitrobenzaldehyde (50 mmol/L) were added, and the mixture thoroughly homogenized by means of an Ultra-Turrax. Then the slurry incubated in a water bath at 37 °C overnight. After cooling, the mixture was neutralized at pH 7.0–7.5 with $K_2HPO_4$ (1 mol/L). Ethyl acetate (8 mL) was added to the slurry before being thoroughly hand-shaked for 2 min, and centrifuged at 2000 × *g* for 3 min at room temperature. The organic phase was collected into a 10-mL Falcon tube and evaporated to dryness under a stream of nitrogen at 40 °C. Then the dry residue reconstituted with distilled 1.0 mL methanol:water (v:v = 20:80). The resulting solution was filtered through a 0.45 μm nylon filter directly into an HPLC vial.

## 3. Results and discussion

### 3.1. Electrochemical characterization of the immunosensor

Electrochemical impedance spectroscopy (EIS) was successfully used as a sensitive method to probe the interface properties of surface-modified electrodes. Its typical impedance spectrum is presented in the form of Nyquist plot, which including a semicircle part and a linear part. The semicircle part at higher frequencies corresponds to the electron-transfer limited process and its diameter is equal to the electron-transfer resistance ($R_{ct}$) which controls the electron-transfer kinetics of the redox probe at the electrode

interface. Impedance measurements have been carried out during the various steps of the antibody immobilization process in 0.1 mol/L PBS (pH 7.5) and 0.1 mol/L $KNO_3$ containing 1.0 mmol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1 mixture) as a redox probe (Fig. 1A). It can be seen that the major changes in the impedance character occurred. At bare GCE, only very small semicircle could be observed, indicating a low transfer resistance (curve a). When the silica sol–gel was modified on the surface of GCE (sol–gel/GCE), the diameter of semicircle increased (curve b). After the mixture of silica sol–gel and AHD-McAb was deposited on the surface of GCE (denoted as sol–gel-AHD-McAb/GCE), the diameter of semicircle was increased again (curve c), implying that the sol–gel-AHD-McAb film obstructed the electron transfer in the electrochemical probe. Then $R_{ct}$ further increased obviously (curve d) after the resulting immunosensor was incubated in $2.0 \times 10^2$ ng/mL AHD, indicating that the immunoreaction between AHD-McAb and AHD occurred and the formation of hydrophobic immunocomplex layer hindered the diffusion of $[Fe(CN)_6]^{4-/3-}$ towards the electrode surface.

An equivalent circuit and fitting of one measured electrochemical impedance spectrum (solid line) were both obtained based on FRA software of Autolab (Fig. 1B), indicating good agreement with the circuit model and the measurement system in the entire measurement frequency range. The equivalent circuit could consist of the solution resistance ($R_s$), the electron-transfer resistance ($R_{ct}$), the Warburg impedance ($Z_w$) and constant phase element ($Q$). Based on the Randles equivalent circuit, the fitting value for the each step of the immunosensor elaboration on the electrode is presented in Table 1. It can be seen that $R_{ct}$ is a suitable signal for sensing the interfacial properties of the prepared immunosensor step by step.

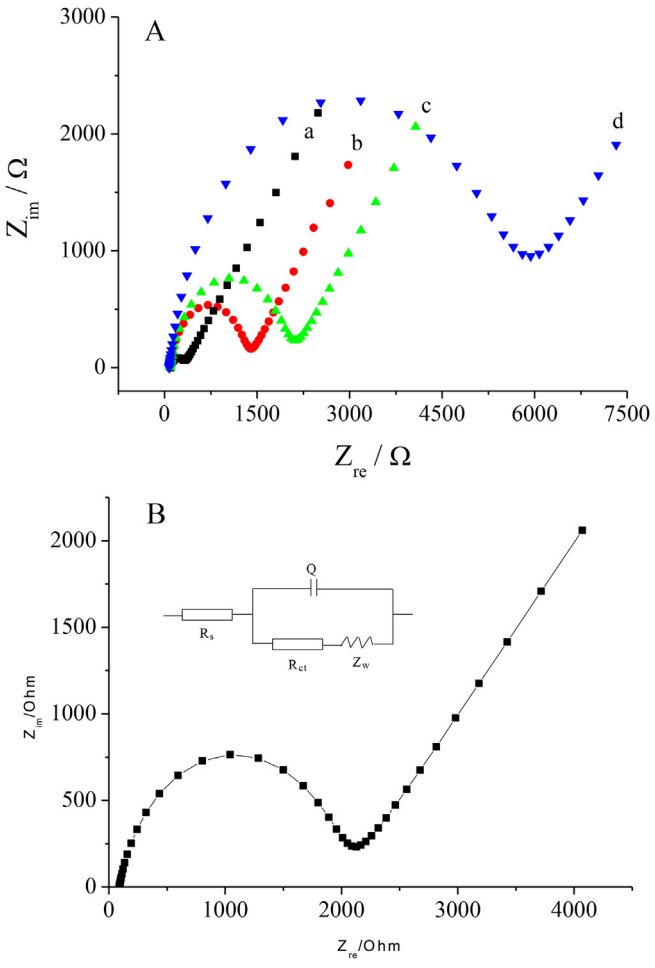



**Fig. 1.** (A) Nyquist plot ($Z_{im}$ vs. $Z_{re}$) for Faradaic impedance measurements in the presence of 0.1 mol/L PBS (pH 7.5) and 0.1 mol/L $KNO_3$ containing 1.0 mmol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at: (a) bare GCE, (b) sol–gel/GCE, (c) sol–gel-AHD-McAb/GCE, and (d) AHD/sol–gel-AHD-McAb/GCE. The frequency range is from $10^{-1}$–$10^6$ Hz with a signal amplitude of 10 mV. (B) Fitted (solid line) and experimental (scattered line) Nyquist plots of impedance spectra. The inset is the equivalent circuit applied to fitted the impedance spectra in the presence of the redox probe of $Fe(CN)_6^{3-/4-}$.

**Table 1**
Randles parameters of the immunosensor elaboration step by step obtained from Fig. 1.

| Electrode | $R_{ct}$/KΩ (SD) | $R_s$/Ω | $n$ | $Q$/F | $Z_w$ |
|---|---|---|---|---|---|
| Bare GCE | 0.40 (0.015) | 139.8 | 0.8967 | $2.498 \times 10^{-7}$ | $2.345 \times 10^{-4}$ |
| Sol–gel/GCE | 1.38 (0.05) | 139.3 | 0.8977 | $2.398 \times 10^{-7}$ | $2.343 \times 10^{-4}$ |
| Sol–gel-AHD-McAb/GCE | 2.0 (0.06) | 139.2 | 0.9011 | $2.276 \times 10^{-7}$ | $2.341 \times 10^{-4}$ |
| AHD/sol–gel-AHD-McAb/GCE | 5.6 (0.15) | 146.2 | 0.8943 | $2.814 \times 10^{-7}$ | $2.785 \times 10^{-4}$ |

### 3.2. Optimization of experimental conditions

The immobilization of antibody on the sol–gel/GCE and interaction between AHD and AHD-McAb could change the interface properties of electrodes, resulting in a change of $R_{ct}$. Several factors, such as immobilization conditions for the amount of AHD-McAb, pH, incubation time and temperature, were investigated in detailed.

The pH value of silica sol–gel would directly affect the activity of embedded antibody. The different pH values of silica sol–gel were adjusted by using 5 mol/L NaOH, and it was mixed with AHD-McAb to maintain its concentration of 0.12 mg/mL, respectively. EIS of sol–gel-AHD-McAb/GCE was recorded in 0.1 mol/L PBS and 0.1 mol/L $KNO_3$ containing 1.0 mmol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (Fig. 2). It can be seen that the highest activity of embedded AHD-McAb in sol–gel can be obtained at pH 7.5 when pH values were from 6.0 to 8.5. Accordingly, an ideal pH of 7.5 of silica sol–gel was chosen for the matrix of antibody immobilization.

The amount of AHD-McAb embedded on the silica sol–gel was optimized (Fig. 3). When the AHD-McAb concentration in silica sol–gel was less than 0.080 mg/mL, the $R_{ct}$ increased with the

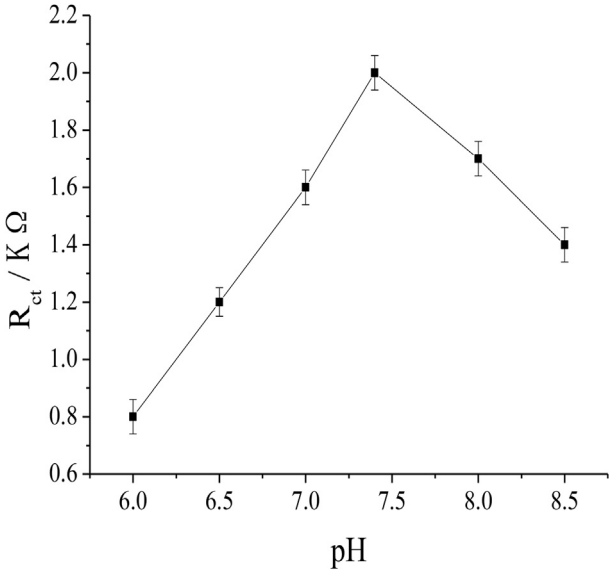



**Fig. 2.** Effect of pH about silica sol–gel on the electrochemical resistance. AHD-McAb concentration in silica sol–gel: 0.12 mg/mL.

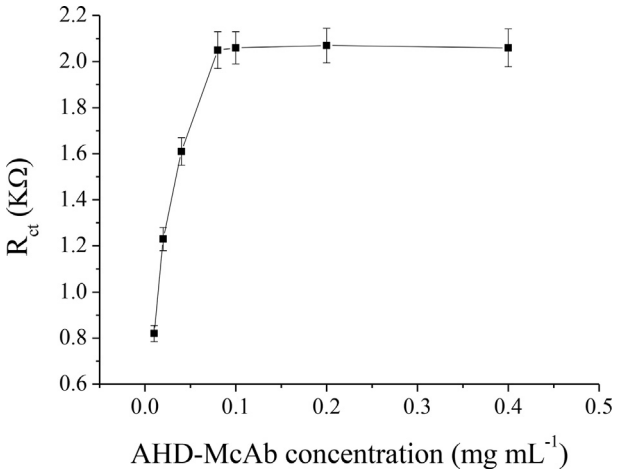



**Fig. 3.** Effect of AHD-McAb concentration in silica sol–gel on the electrochemical resistance.

increasing its concentration. And then the $R_{ct}$ reaches a stable value when the antibody concentration was in the range from 0.080 to 0.40 mg/mL. Thus, the 0.012 mg/mL of AHD-McAb in sol–gel was selected in the following experiment.

It is well known that the pH of the solution has a profound effect on the immunosensor. The effect of pH in the immunoreaction between AHD and AHD-McAb was studied in the different pH value of 0.1 mol/L PBS and 0.1 mol/L $KNO_3$ (Fig. 4A). It can be seen that the change of resistance ($\Delta R_{ct}$) increased with the increase of pH value from 6.0 to 7.5, and then decreased when pH was in the range of 7.5–8.5. Thus, pH 7.5 of 0.1 mol/L PBS was selected as the buffer solution in the binding reaction.

The effect of incubation temperature on $\Delta R_{ct}$ was also investigated in the temperature range from 20 to 50 °C (Fig. 4B). As shown in Fig. 4B, the $\Delta R_t$ increased with increasing temperature up to 37 °C, and then it decreased while temperatures was over 37 °C. The reason may be that the high temperature caused an irreversible behaviour (denaturation of proteins) of the AHD-McAb and AHD in the process. Thus, the incubation temperature of 37 °C was used as the optimum incubation temperature.

Immunoreaction time played an important role in the detection of AHD. In the immunoreaction solution, when the AHD molecules reach the antibodies immobilized on the electrode surface of the immunosensor, it takes some time for the contacting species to form immunocomplex. The influence of the immunoreaction time on $\Delta R_{ct}$ is investigated (Fig. 4C). When the immunoreaction time was less than 150 min, the $\Delta R_{ct}$ was increased with the increasing of time, while $\Delta R_{ct}$ hardly changed when the immunoreaction time was in the range of 150–180 min. It manifested that the adsorptive immobilization process of AHD reached the saturated equilibrium after the immunoreaction between AHD-McAb and AHD for 150 min. Thus, the immunoreaction time of 160 min was selected for the whole immunoassay.

### 3.3. Performance of the immunosensor

A Nyquist diagram of the electrochemical impedance spectrum is an effective way to measure the electron-transfer resistance. Fig. 5A shows the Nyquist diagrams of the proposed immunosensor immunoreacted with different AHD concentrations. The electron-transfer resistance increased with the increase of AHD concentrations as shown in curves b–f. It may be due to more AHD molecules binding to the immobilized antibodies in higher AHD concentrations, which acts as a definite kinetic barrier for the

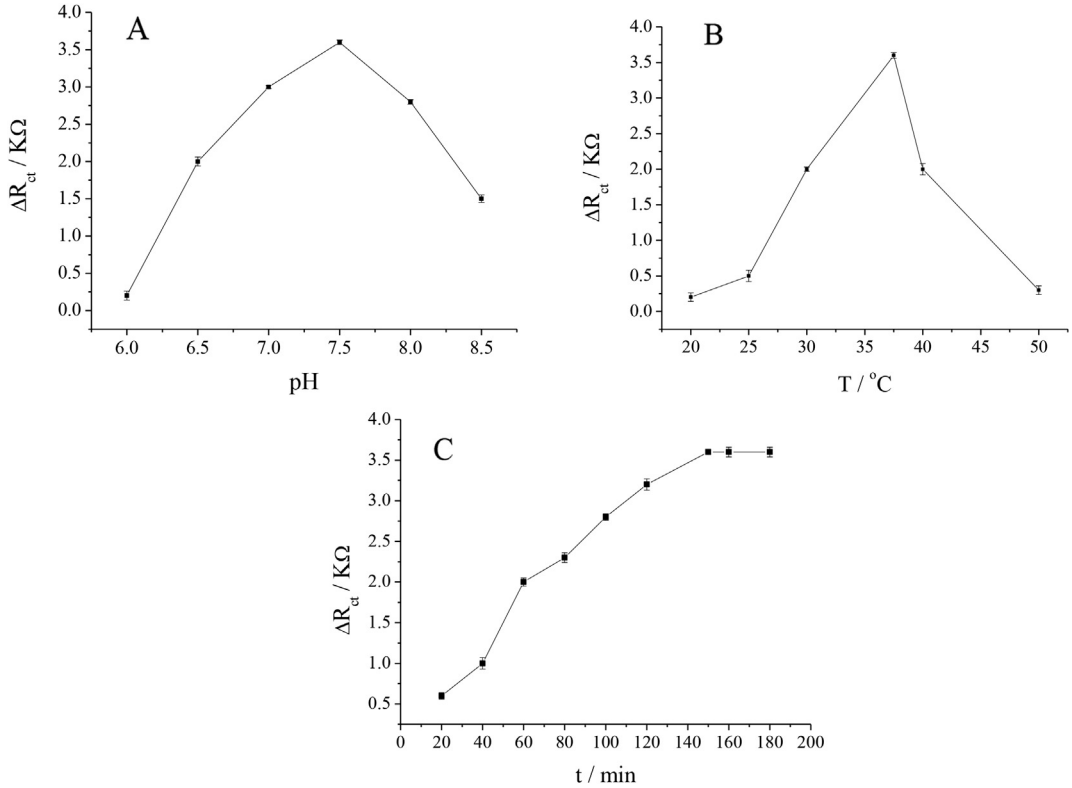



**Fig. 4.** The influence of the incubation pH (A), incubation temperature (B), and incubation time (C) between AHD-McAb and AHD interaction. The concentration of AHD was $2.0 \times 10^2$ ng/mL.

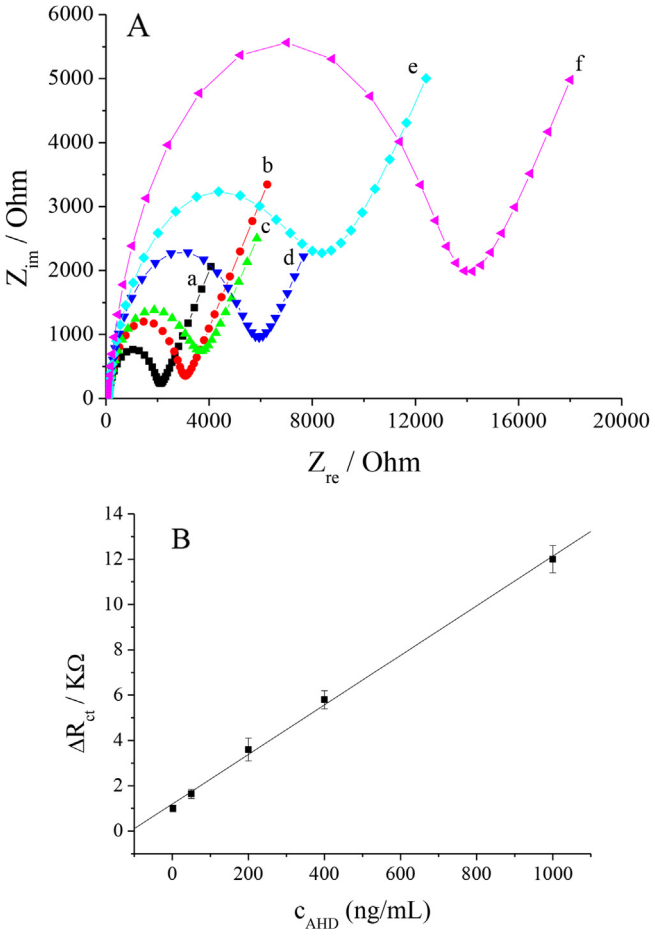



**Fig. 5.** (A) Faradaic impendence spectra that the immunosensor incubated with different concentrations of AHD in 0.1 mol/L PBS (pH 7.5) and 0.1 mol/L $KNO_3$ containing 1.0 mmol/L $Fe(CN)_6^{4-/3-}$: curves a–f represent 0, 2.0, 50.0, $2.0 \times 10^2$, $4.0 \times 10^2$, $1.0 \times 10^3$ ng/mL AHD, respectively. (B) Calibration curve for the immunosensor.

**Table 2**
Change in impedance for the proposed immunosensor in 2.0 ng/mL AHD containing different concentrations of interfering metabolites of nitrofurans.

| Concentration (ng/mL) | Interfering compounds | | | | | |
|---|---|---|---|---|---|---|
| | AMOZ | | AOZ | | SEM | |
| | $R_{ct}$ ± SD (KΩ) | RSD | $R_{ct}$ ± SD (KΩ) | RSD | $R_{ct}$ ± SD (KΩ) | RSD |
| 0 | 1.0 ± 0 0.032 | 3.2 | 1.1 ± 0.037 | 3.4 | 1.0 ± 0.034 | 3.4 |
| 10.0 | 1.1 ± 0.041 | 3.7 | 1.2 ± 0.051 | 4.2 | 1.1 ± 0.43 | 3.2 |
| $1.0 \times 10^2$ | 1.1 ± 0.043 | 3.9 | 1.2 ± 0.047 | 3.9 | 1.0 ± 0.041 | 4.1 |
| $1.0 \times 10^3$ | 1.1 ± 0.046 | 4.2 | 1.1 ± 0.042 | 3.8 | 1.1 ± 0.048 | 4.4 |

electron transfer. Under optimum conditions, the calibration curve of the immunosensor to different concentrations of AHD is shown in Fig. 5B, which exhibited a good linear relationship between $\Delta R_{ct}$ and AHD concentration in the range from 2.0 to $1.0 \times 10^3$ ng/mL. The linear regression equation was represented as $\Delta R_{ct} = 1.19 + 0.0109 C_{AHD}$(ng/mL) with a correlation coefficient of 0.9990 and the detection limit of 2.0 ng/mL (Chen et al., 2008). Based on the linear equation, the AHD content in food samples could be determined quantitatively.

The reproducibility of the proposed immunosensor was evaluated by analyzing AHD level for six replicate measurements. The intra-assay coefficients of variation with the above method were 3.7% and 3.3% at AHD concentrations of 10.0 and $2.0 \times 10^2$ ng/mL, respectively. The fabrication reproducibility was evaluated using six immunosensors made independently at the same electrode. The relative standard deviation (RSD) of the measurements was 5.9% and 5.5% at AHD concentrations of 10.0 and $2.0 \times 10^2$ ng/mL, respectively. The above experimental results indicate that the proposed immunosensor has the good precision and reproducibility.

The stability of the immunosensor was examined by storage at 4 °C when not in use. The $\Delta R_{ct}$ was tested at the same AHD concentration of $1.0 \times 10^2$ ng/mL every 3 days. No obvious change was observed after a 14-day storage, and then decreased gradually and retained 81.1% of initial value after one month storage. The good stability of proposed immunosensor may be contributed to following some factors. First, the microstructure of modified electrode hardly changed. Secondly, AHD-McAb can be well embedded and maintain its good biological activity in silica sol–gel.

The selectivity of the immunosensors was also investigated. The other three metabolites of nitrofurans, such as AMOZ, AOZ, and SEM, were tested under the condition of 2.0 ng/mL AHD, and the relative change in $R_{ct}$ was calculated before and after the different amount of interfering compounds was added (Table 2). The $R_{ct}$ variation was less than 4.4% with the increase of AMOZ, AOZ, and SEM concentrations, respectively, indicating that the selectivity of the proposed immunosensor was acceptable. These results clearly confirmed that the observed relative change in impedance originated from specific interaction between AHD-McAb and AHD.

### 3.4. Preliminary analysis of real samples

In order to evaluate the feasibility of the proposed immunosensor for real sample analysis, the immunosensor was used for the determination of AHD residue by standard addition methods in some food samples of pork floss, shrimp, honey, pig bladder, salted hog casings, and crab. Under optimum experimental conditions, the content of AHD in the above samples was detected. At the same time, the HPLC–MS/MS was used as a reference method which was offered by EU. Now it was widely accepted to detect nitrofurans' residues in food samples by many countries. The comparison

**Table 3**
Comparative determination of AHD in food samples by the proposed method and HPLC–MS/MS method.

| Samples | Amount found ± SD (mg/kg) | | $F$-value[b] $F = S_1^2/S_2^2 (s_1 > s_2)$ | $t$-Value[c] $t = \lvert\overline{X}_1 - \overline{X}_2\rvert / S\sqrt{n_1 n_1/n_1 + n_2}$ |
|---|---|---|---|---|
| | Method based on impedimetric immunosensor ($n = 5$) | HPLC–MS/MS ($n = 4$) | | |
| Pork floss | ND[a] (<1 μg/kg) | ND (<1 μg/kg) | / | / |
| Shrimp | 0.31 ± 0.01 | 0.29 ± 0.01 | 1 | 2.98 |
| Honey | ND (<1 μg/kg) | ND (<1 μg/kg) | / | / |
| Pig bladder | ND (<1 μg/kg) | ND (<1 μg/kg) | / | / |
| Salted hog casings | ND (<1 μg/kg) | ND (<1 μg/kg) | / | / |
| Crab | 0.26 ± 0.02 | 0.28 ± 0.03 | 2.25 | 1.14 |

[a] ND: not detected.
[b] $F$-values at 99% confidence level are as follows: for $n_1 = 5$, $n_2 = 4$, $F = 9.12$.
[c] $t$-Values at 99% confidence level are as follows: for $n_1 = 5$, $n_2 = 4$, $t = 3.50$.

**Table 4**
The recovery of food samples by the proposed method obtained.

| Samples | Added (ng/mL) | Founded (ng/mL) | Recovery (%) | RSD (%) |
|---|---|---|---|---|
| Pork floss | 10.0 | 9.37 | 93.7 | 5.2 |
| | $1.00 \times 10^2$ | $1.032 \times 10^2$ | 103.2 | 4.1 |
| | $1.00 \times 10^3$ | $9.81 \times 10^3$ | 98.1 | 3.6 |
| Shrimp | 10.0 | 9.65 | 96.5 | 4.8 |
| | $1.00 \times 10^2$ | $9.86 \times 10^2$ | 98.6 | 3.7 |
| | $1.00 \times 10^3$ | $1.013 \times 10^3$ | 101.3 | 4.4 |
| Honey | 10.0 | 10.41 | 104.1 | 4.3 |
| | $1.00 \times 10^2$ | $9.55 \times 10^2$ | 95.51 | 4.1 |
| | $1.00 \times 10^3$ | $9.733 \times 10^3$ | 97.33 | 3.4 |
| Pig bladder | 10.0 | 9.51 | 95.1 | 4.5 |
| | $1.00 \times 10^2$ | $9.71 \times 10^2$ | 97.1 | 4.1 |
| | $1.00 \times 10^3$ | $1.047 \times 10^3$ | 104.7 | 3.4 |
| Salted hog casings | 10.0 | 10.49 | 104.9 | 3.1 |
| | $1.00 \times 10^2$ | $9.62 \times 10^2$ | 96.2 | 3.9 |
| | 1.00c | $9.84 \times 10^3$ | 98.4 | 3.9 |
| Crab | 10.0 | 9.81 | 98.1 | 4.6 |
| | $1.00 \times 10^2$ | $1.019 \times 10^2$ | 101.9 | 4.0 |
| | $1.00 \times 10^3$ | $9.49 \times 10^3$ | 94.9 | 3.8 |

between impedimetric immunosensor and HPLC results were shown in Table 3, which was in good agreement between the two methods. Based on analyzed statistically (*t*-test), there is no significant difference between two methods.

In order to manifest the accuracy of the proposed method, the recovery was carried out. The recovery was studied by spiking these samples with three AHD levels at 10.0, $1.0 \times 10^2$ and $1.0 \times 10^3$ ng/mL, and the results were shown in Table 4. The satisfied recoveries were obtained in the range of 93.7–104.9% in six food samples, which indicated that the proposed accurate method could be satisfactorily applied to detect the AHD residue in food samples.

## 4. Conclusions

In this paper, we reported an AHD-McAb-based impedimetric immunosensor for the determination of AHD residue in food samples. Silica sol–gel hybrid material provided an excellent matrix for the immobilization of AHD-McAb, and its networks not only effectively entrapped the antibody molecule, but also increased the long-term stability of immobilized antibody. The antibody–antigen affinity reaction resulted in an extremely sensitive specific impedance response, and could be sensitively and selectively quantified by the change in $R_{ct}$ ($\Delta R_{ct}$) with AHD concentration as low as 2.0 ng/mL. Compared to the method of HPLC–MS/MS, the proposed method performed some merits, such as the short whole analysis time and the simple sample's treatment due to omitting the tedious extraction, clean-up steps and derivation prior to chromatography. The experimental results showed that the proposed method was applied to the determination of AHD in food samples with satisfactory results. Most importantly, it is expected that such combined strategies would be useful for the development of electrochemical immunosensors for the detection of other analytes.

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