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Food safety control of α-zearalanol through voltammetric immunosensing on Au-Pt bimetallic nanoparticles surface

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Abstract

This study reports an accurate and sensitive strategy for *α-zearalanol* (ZER) determination in bovine urine samples. ZER is a mycotoxin widely used as synthetic growth promoter in the livestock production whose residues could present a potential risk for human health. Therefore, the use of it as animal feed additive has been banned in most countries. ZER determination was accomplished using an electrochemical system in which bimetallic Au-Pt nanoparticles (Au-PtNPs) were electro-synthesized on screen printed carbon electrode (SPCE). The obtained Au-PtNPs platform was immunofunctionalized with specific anti-ZER antibodies as a strategy to avoid potential interferences. After biorecognition, ZER was directly oxidized and detected by square-wave voltammetry (SWV). The Au-PtNPs surface was characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD) and cyclic voltammetry (CV). The limit of detection calculated was 0.01 ng mL^{-1} with a wide linear range from 0.03 to 30 ng mL^{-1}. This method promises to be suitable for ZER quantification in bovine urine samples ensuring food quality and safety, as well as consumer's health.

1. Introduction

α-zearalanol (ZER) is a resorcylic acid lactone (RAL),[1-3] derived from zearalenone (ZEO) (Fig. 1), a mycotoxin produced by *Fusarium spp*.[4,5] It is one of the compounds widely used as synthetic growth promoters to improve feed efficiency in the livestock production.[6-10] The use of this anabolic agent in animals may leave harmful residues with long half-life and stability in tissues intended for human consumption. These residues could present a potential risk for human health if they remain in the meat products.[11] Moreover, ZER is considered as an endocrine-disrupting compound (EDC) due to its high estrogen receptor binding affinity and estrogenic potency.[12-14]

EDCs are any environmental pollutant chemical that, once incorporated into an organism, affects the hormonal balance of various species including humans. Furthermore, EDCs are potentially involved in the increasing incidence of cancer in breast and testis in humans.[15,16] Therefore, the use of this drug as animal feed additives has been banned in most countries.[17] The establishment of regulatory measures for the use of this growth promoter in animal husbandry requires the availability of sensitive analysis methods to achieve the determination of ZER for screening and confirmation.

To date, various analytical methods have been reported for RALs determination in many biological tissues and fluids, including gas chromatography–mass spectrometry,[18,19] liquid chromatography-mass spectrometry,[20,21] ultra-high-pressure liquid chromatography-tandem mass,[22] capillary electrophoresis.[23] These methods present important analytical benefits such as high sensitivity and selectivity. However, they require expensive instruments and long samples pretreatment. Thus, it is crucial to develop an alternative, simple and fast method for the analysis of ZER in bovine urine samples. For this reason, and taking into account that this drugs family can be oxidized because of the presence of an

electroactive group in its chemical structure, electrochemical methods represent an attractive strategy for ZER determination.

An interesting electrochemical approach can involve the use of bimetallic nanoparticles electro-synthetized on SPCE as the basis for the bioaffinity reaction and transduction step. Bimetallic nanoparticles have gained great relevance for both scientific and technological perspective because of their outstanding optic, catalytic, mechanical and electric properties generated by the combination of two kinds of metals. These properties are distinct from those corresponding to the monometallic counterparts.[24-27] Among the bimetallic nanoscale materials the Au-PtNPs have been reported in literature as a promising material for electrode modification, showing advantages over monometallic components, such as better sensitivity and longer stability of electrode systems.[28-33] The functionalization of NPs surface with immunoreagents allowed to obtain a bioaffinity support with high selectivity as a consequence of the antigen-antibody binding specificity.[34-36]

To the best of our knowledge, no study involving bimetallic Au-Pt nanoparticles electro-synthesized on SPCE as platform for the immunorecognition of ZER and its subsequent determination by SWV has been reported. In this article, we present and discuss an accurate, sensitive and selective method for ZER determination in bovine urine samples. Here, ZER is recognized by specific anti-ZER antibodies immobilized on Au-PtNPs/SPCE in order to achieve the selective determination avoiding potential interferences.

2. Experimental

2.1 Reagents and solutions

All reagents were of analytical grade. ZER, K_2PtCl_6, $HAuCl_4$, β-glucuronidase from *Escherichia coli*, 3-mercaptopropionic acid (MPA), N-(3-dimethylaminopropyl)-N-

ethylcarbodiimide (ECD), bovine serum albumin (BSA) and N-hydroxysuccinimide (NHS) were acquired from Sigma-Aldrich, St. Louis, USA. Disodium phosphate (Na_2HPO_4), monosodium phosphate (NaH_2PO_4), potassium chloride (KCl) and ethanol were purchased from Merck, Darmstadt, Germany. Rabbit anti-ZER antibody was obtained from CER GROUP, Marloie, Belgium. ZER ELISA KIT was supplied by RANDOX (Diamond Road, United Kingdom). All solutions were prepared by using water purified in a Barnstead Easy pure RF compact ultra-pure water system. The pH values of the buffer solutions were determined with an Orion Expandable Ion Analyzer, model EA 940 equipped with a glass combination electrode (Orion Research Inc, Cambridge, USA). Working standard solutions were daily prepared by appropriate dilution of the stock solutions with deionized water.

2.2 Instruments and measurements

Scanning electron micrographs were obtained using a LEO 1450VP SEM (Labmem, San Luis, Argentina). The elemental composition of bimetallic nanoparticles was determined by XRD using a Rigaku D-MAX IIIC diffractometer with copper radiation (ka = 0.154178 nm) and a nickel filter (Rigaku, Texas, USA). CV and SWV measurements were performed using a BAS 100 B/W (electrochemical analyzer Bioanalytical System, West Lafayette, IN, USA). A SPCE with three electrodes was used. SPCE was composed by a silver ink (Ag) as pseudo-reference, graphite ink as auxiliary and a graphite circular ink (Ø = 3 mm). The applied potentials were referred to Ag pseudo-reference electrode. The solutions and reagents were conditioned at room temperature before the experiments using a laboratory water bath (Vicking Mason II, Vicking SRL, Argentina). Absorbance measurements were carried out by a Bio-Rad Benchmark microplate reader (Bio-Rad, Japan) and Beckman DU 520 general UV/vis spectrophotometer (Beckman, Brea, USA).

2.3 Preparation of Au-PtNPs/SPCE

Before the electrodeposition procedure, the working electrode was pretreated to oxidize the graphite impurities to obtain a more hydrophilic surface.[37,38] To obtain the electrodeposited PtNPs onto the SPCE, the following procedure was employed. The SPCE was immersed into 500 μL of platinum solution (K_2PtCl_6) 0.2% containing 0.01 mol L^{-1} KCl as supporting electrolyte. After that, a constant potential of -0.4 V (vs Ag-SPCE) was applied for 60 s (S1). Then, the PtNPs/SPCE was rinsed by mechanically stirring with double distilled water and dried carefully with pure nitrogen gas. In order to electrodeposit Au onto the PtNPs, the procedure described by Pereira et al. was adapted.[32] The PtNPs/SPCE was immersed into 500 μL of gold solution ($HAuCl_4$) 0.1% containing 0.01 mol L^{-1} KCl as supporting electrolyte. After, a constant potential of -0.2 V (vs Ag-SPCE) was applied for 60 s. The obtained Au-PtNPs/SPCE was rinsed and dried as mentioned above.

2.4. Preparation of anti-ZER/Au-PtNPs/SPCE

Au-PtNPs/SPCE was placed in MPA 0.04 mol L^{-1} in 3:1 (v/v) EtOH/H_2O solution for 15 h at room temperature. At this point, the -SH groups of MPA reacted with the surface of Au previously electrodeposited, leaving free -COOH groups, which were activated by rinsing in a solution containing ECD and NHS in 0.05 mol L^{-1} PBS pH 7.20 and then, evaporated to dryness. After that, the electrode was rinsed with double distilled water and dried with pure nitrogen gas. Then, anti-ZER antibodies were immobilized onto the Au-PtNPs/SPCE. For this step, the Au-PtNPs/SPCE was exposed to anti-ZER antibody solution (1:500 in 0.01 mol L^{-1} PBS pH 7.20) overnight at 4 ºC. The concentration of anti-

ZER antibody solution represents an important parameter which was previously optimized. Finally, the immunosensor was rinsed with 0.01 mol L^{-1} PBS pH 7.20 and stored in the same solution at 4 ºC when not in use. Schematic representation of the construction of the immunosensor can be visualized in Scheme 1 A).

2.5 Sample preparation

Bovine urine samples from six different specimens were collected, frozen and stored until sample preparation process. As a first step, urine samples were diluted 6-fold with 0.01 mol L^{-1} PBS pH 7.20. This dilution with PBS did not affect the analytical determination. Secondly, 10 µL of β-glucuronidase from *Escherichia coli* were added to diluted samples and incubated for 3 h at 37 °C (alternatively over night at room temperature) in order to produce hydrolysis of complex ZER-glucuronide. Finally, the removal of fluid residues by positive pressure or vacuum was carried out. Samples were stored at 4 °C and protected against light until analysis. In order to verify the accuracy of ZER voltammetric determination with anti-ZER/Au-PtNPs/SPCE, the results were compared to those obtained by the commercial enzyme immunoassay.

2.6 ZER voltammetric determination with anti-ZER/Au-PtNPs/SPCE

The proposed method was applied to ZER determination in bovine urine samples. Initially, the nonspecific bindings were blocked by a 2 min treatment with 50 µL of 1 % BSA at room temperature. After that, 50 µL of bovine urine sample previously pretreated were placed on the anti-ZER/Au-PtNPs/SPCE surface for 5 min. In this step, the ZER present in the sample reacted specifically with the immobilized anti-ZER antibodies. Later, 50 µL of desorption buffer (0.1 mol L^{-1} citrate pH 2.0) were added for 2 min. Between each

step, the immunosensor was rinsed with 0.01 mol L^{-1} PBS pH 7.20. Finally, ZER desorbed in the previous step was oxidized at +1.124 V (vs Ag-SPCE) by SWV. The current response was directly proportional to the amount of the ZER in the sample. The blank solution was prepared in the same way as samples, except that 50 μL of bidistilled water were used instead of the sample solution. Schematic representation of ZER determination with anti-ZER/Au-PtNPs/SPCE can be visualized in Scheme 1 B).

3. Results and discussion

3.1 Characterization of the Au-PtNPs/SPCE

Electrochemical deposition method was chosen to obtain NPs because size, shape and composition of nanoparticles are controlled by varying the composition of electrolyte solutions, current density and potentials during the deposition process.[39]

NPs obtained by electrochemical deposition method have been characterized with SEM, XRD and CV. By analyzing the SEM images shown in Fig. 2 (A–C) the contribution of each material used in the construction of the Au-PtNPs/SPCE can be visualized. Fig. 2 (A) reveals the images of SPCE without modifications. Fig. 2 (B) shows that PtNPs are well distributed on the SPCE. These NPs had a size of 25 $\pm$ 5 nm in diameter. Fig. 2 (C) reveals that Au was distributed homogeneously over the previously electrodeposited PtNPs. The growth of Au around the already deposited Pt seeds can be observed. The diameter of these bimetallic nanoparticles was 45 $\pm$ 5 nm. Electrochemical deposition of metallic nanoparticles results in appearance of small particles on the graphite electrode, obtaining in this way a large surface area. The electrodes employed in the characterization step were previously optimized as shown in Section 2.3.

The crystalline structure of Au-PtNPs/SPCE was characterized by XRD measurement. The XRD pattern is shown in Fig. 2 (D). The peaks at 2θ (37.2 and 37.5º) resulted from Au and the peak at 2θ (38.2º) was from Pt. The average size of the crystalline structure of the deposited Au-PtNPs was calculated according to the Scherrer equation ($t = K \times \lambda / B \times \cos \theta$) and the obtained value was approximately 45 nm.

Figure 3 shows the electrochemical response of the SPCE after each modification step. CVs of the redox probe $Fe(CN)_6^{4-/3-}$ represent a convenient and valuable tool to monitor the characteristics of the modified electrode surface. CVs of SPCE (—), AuNPs/SPCE (—), PtNPs/SPCE (—) and Au-PtNPs/SPCE (—) were carried out in 1 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in 0.1 mol L^{-1} KCl (pH 6.50) solution. The potential sweep was performed from -0.2 V to +0.8 V (vs Ag-SPCE) at a scan rate of 75 mV s^{-1}. Well-defined cyclic voltammograms and characteristics of a diffusion-controlled redox process were observed at the bare SPCE surface (—). As shown in curve (—) and (—), when the SPCE was modified with AuNPs and PtNPs, the peak current enhanced greatly due to NPs increase the effective surface area. The subsequent Au electrodeposition over PtNPs resulted in Au-PtNPs/SPCE which exhibited a highest current response due to the increase in the effective surface area (—).

The stability of the Au-PtNPs/SPCE under successive scans was also monitored. The modified electrode was scanned successfully for 20 cycles in a 0.1 mol L^{-1} KCl (pH 6.50) solution, containing 1 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ at 75 mV s^{-1}, and no observable changes in peak current were found, indicating that the sensor had good stability (data not shown).

3.2 Electrochemical behaviour of ZER on anti-ZER/Au-PtNPs/SPCE

CV obtained in a 0.1 mol L^{-1} citrate solution at pH 2.0 containing methanol 10% at 75 mV s^{-1} in the absence and presence of 1 mmol L^{-1} ZER are shown in Fig. 4. As can be seen, in the presence of ZER, an anodic peak at E_{pa} = +1.124 V corresponding to a surface process was observed. Besides, the CV shows an irreversible behaviour, which was evidenced by the absence of the corresponding cathodic peak when the direction of potential sweep was reversed.

3.3 Analytical characterization

In order to establish the optimum conditions, the electrochemical signal of a ZER standard concentration of 3.5 ng mL^{-1} was directly monitored by SWV using anti-ZER/Au-PtNPs/SPCE. Regarding to voltammetric determination, SWV specific parameters were evaluated. The optimum conditions were: step E = 4 mV, S.W. amplitude = 25 mV, S.W. frequency = 15 Hz, samples per point = 256, studied potential range = 0–+1.400 V, sensitivity = 10 μA V^{-1}.

As mentioned before, the ZER bound to the electrode surface was desorbed in acidic solution. The influence of pH and concentration of the desorption solution were evaluated. This fact is due to these variables have effect about the reaction efficiency of the antigen–antibody complex. The response of the modified electrode was investigated in the pH range from 1.00 to 7.00. The maximum electrochemical response was obtained at pH 2.0. When it increased the signal was reduced showing that the desorption process of the ZER-anti-ZER complexes was less efficient Fig. 5 (A).

The effect of the desorption solution concentration on the voltammetric response of the modified electrode was examined from 0.025 at 0.15 mol L^{-1}. The response of the

electrode increased up to 0.1 mol L^{-1} and remained constant for higher values. Therefore, the solution concentration of 0.1 mol L^{-1} was selected for the experiments Fig. 5 (B).

The effect of the volume on the voltammetric determination of ZER was studied using solution volumes from 10 to 100 µL containing 0.1 mol L^{-1} citrate (pH 2.0). The results showed that the measured current increased between 10 and 50 µL. This volume of desorption solution cleaves the antigen–antibody complex on the SPCE. Later, the measured current decreased when solution volume size up to 100 µL, since the solution was diluted. Therefore, a volume of 50 µL was selected as optimum value Fig. 5 (C).

3.4 Analytical parameters

Voltammetric responses displayed employing anti-ZER/Au-PtNPs/SPCE after successive additions of ZER in 0.1 mol L^{-1} citrate buffer at pH 2.0 containing methanol 10% are shown in Fig. 5 (D). As can be seen in the plot, a linear response range from 0.03 to 30 ng mL^{-1} was obtained. It can be expressed according to the following equation: ΔI (nA) = 188.07 + 27.21 [ZER] (ng mL^{-1}), with a correlation coefficient of 0.996 (for n = 5), where ΔI is the difference between current of the blank and sample. The detection (LOD) and quantification (LOQ) limits were determined according to the IUPAC recommendations,[40] achieving values of 0.01 and 0.03 ng mL^{-1}, respectively.

The reproducibility of the voltammetric immunosensor was evaluated in 0.1 mol L^{-1} citrate at pH 2.0 measuring the current (ΔI) by SWV in the presence of 3.5 ng mL^{-1} ZER. For this a series of five anti-ZER/Au-PtNPs/SPCE were prepared in the same way and evaluated employing the same conditions described above showing a relative standard deviation (R.S.D.) value of 5.8% (for n = 5).

The stability of the voltammetric immunosensor was tested checking the current responses. Firstly, when the immunosensor was prepared and not in use, it was stored in the conditions previously described in section 2.4. The current response of the immunosensor was evaluated after 7 and 23 days of storage in PBS at pH 7.20, showing a decrease of 4% and 90% of its initial value, respectively. Therefore, the molecules can be firmly immobilized on the surface of electrode and showed long life time. The slow decrease in the current response can be attributed to the gradual denature of antibodies.[41]

Table 1 compares the results obtained in this work with other papers involving modified electrodes with different electroanalytical techniques for ZER determination. As can be seen, our system is the first electrochemical immunosensor for ZER determination which uses as detection platform a portable SPCE modified with bimetallic Au-PtNPs. Moreover, it employs SWV as detection technique, which offers as chief advantages great sensitivity and fast speed. The previously mentioned features are combined with the selectivity offered by using immunological reagents. Regarding to the LOD, the obtained value allowed us the detection of ZER at very low levels. Finally, is important to highlight the wide linear range achieved.

3.5 Determination of ZER in bovine urine samples

In order to evaluate the analytical applicability, the proposed immunosensor was applied to ZER voltammetric determination in six bovine urine samples (n = 5) under the conditions previously described. The total assay time for the determination of the ZER concentration was 15 min. The ZER concentrations were obtained using the standard addition method and the results were confirmed by enzyme immunoassay using the paired

t-test. The results demonstrated that both methods were statistically equal at a confidence level of 95% (Table 2).

4. Conclusions

The developed anti-ZER/Au-PtNPs/SPCE allowed the determination of ZER in bovine urine samples. Our system showed great sensitivity and selectivity. These two relevant analytical features were achieved through the increase in the surface area generated by electrosynthetized NPs and specificity of antigen-antiboby reaction, respectively. Moreover, the described voltammetric immunosensor employed SWV as detection technique, which offers as chief advantages a great sensitivity and fast speed.

Bimetallic NPs platform was synthetized by an electrodeposition process in which the growth of Au around the already deposited Pt seeds could be observed. The developed method showed many advantages like portability, low cost, wide linear range, accuracy with excellent LOD. The total assay time was 15 min, less than the required by commercial ELISA test. The proposed method could be a very promising analytical tool for the direct determination of ZER in real samples, ensuring safety and quality of food, as well as consumer's health.

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Figure captions

Scheme 1. A) Schematic preparation of anti-ZER/Au-PtNPs/SPCE. B) Schematic representation of ZER determination employing anti-ZER/Au-PtNPs/SPCE.

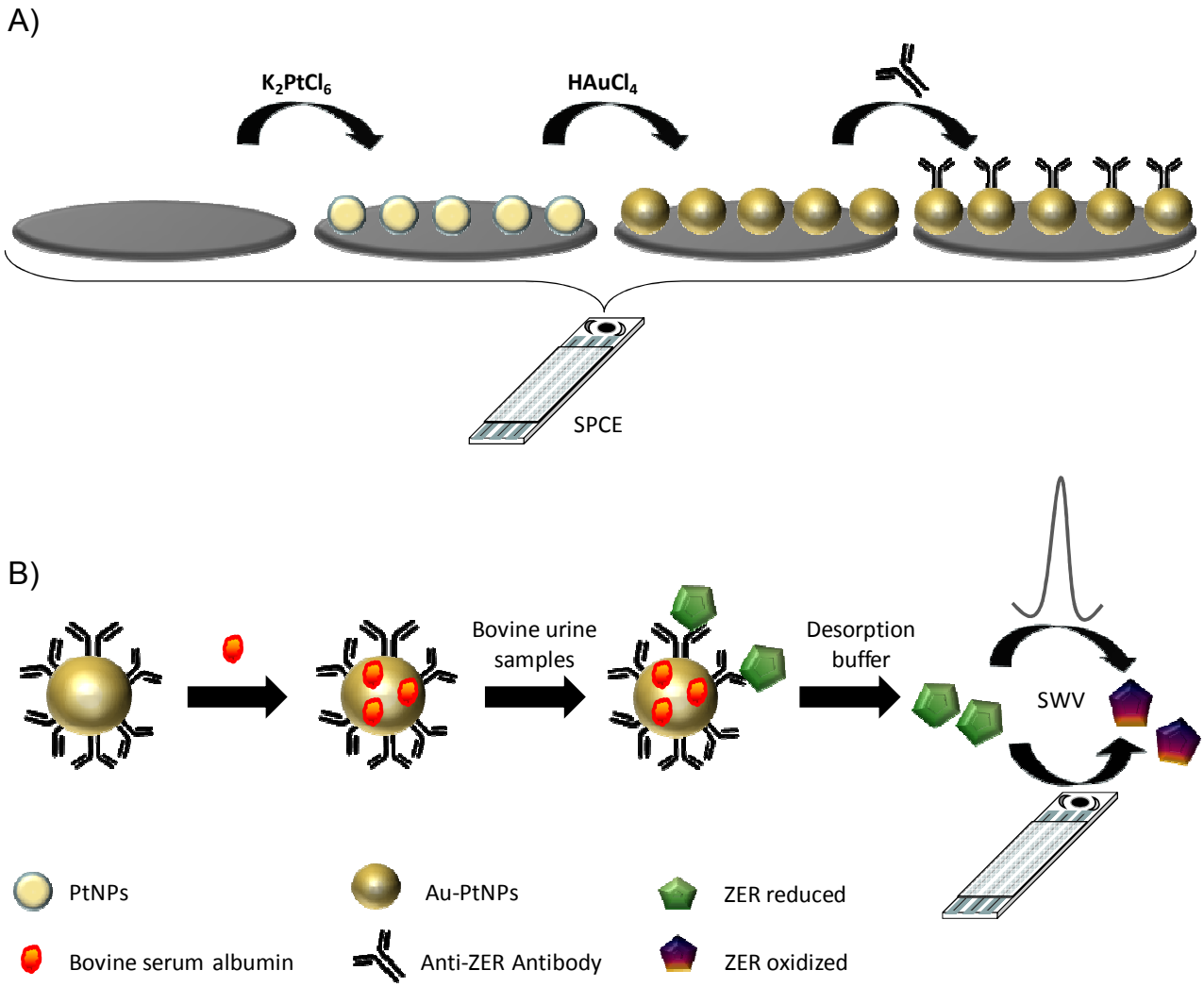
Figure 1. Chemical structures of ZEO (A) and ZER (B).

Figure 2. Characterization of Au-PtNPs/SPCE. Images of A) SPCE, B) PtNPs/SPCE and C) Au-PtNPs/SPCE by SEM. D) Elemental composition of the Au-PtNPs/SPCE by XRD.

Figure 3. Electrochemical characterization of the Au-PtNPs/SPCE. CVs were performed in 1 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in 0.1 mol L^{-1} KCl pH 6.50 solution from -0.2 to +0.8 V at a scan rate of 75 mV s^{-1}.

Figure 4. Electrochemical behavior in the absence and presence of 1 mmol L^{-1} ZER in 0.1 mol L^{-1} citrate solution pH 2.0 containing methanol 10% on anti-ZER/Au-PtNPs/SPCE.

Figure 5. Optimization of experimental conditions. A) pH of citrate solution range from 1.00 to 7.00, B) concentration of citrate solution examined from 0.025 at 0.15 mol L^{-1} and C) volume of citrate solution studied from 10 to 100 µL. D) calibration curve range from 0.03 to 30 ng mL^{-1}, ΔI (nA) = 188.07 + 27.21 [ZER] (ng mL^{-1}), with a correlation coefficient of 0.996 (for n = 5).



Scheme 1

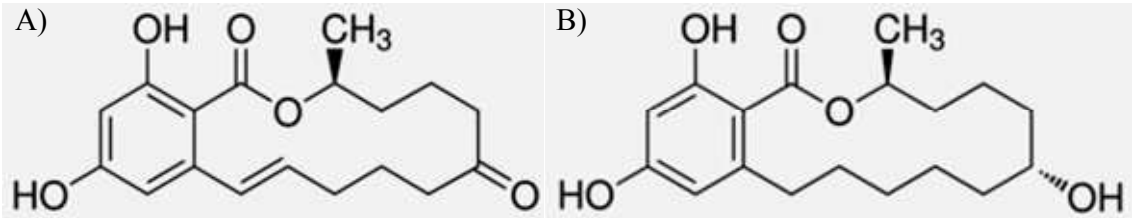


Fig. 1

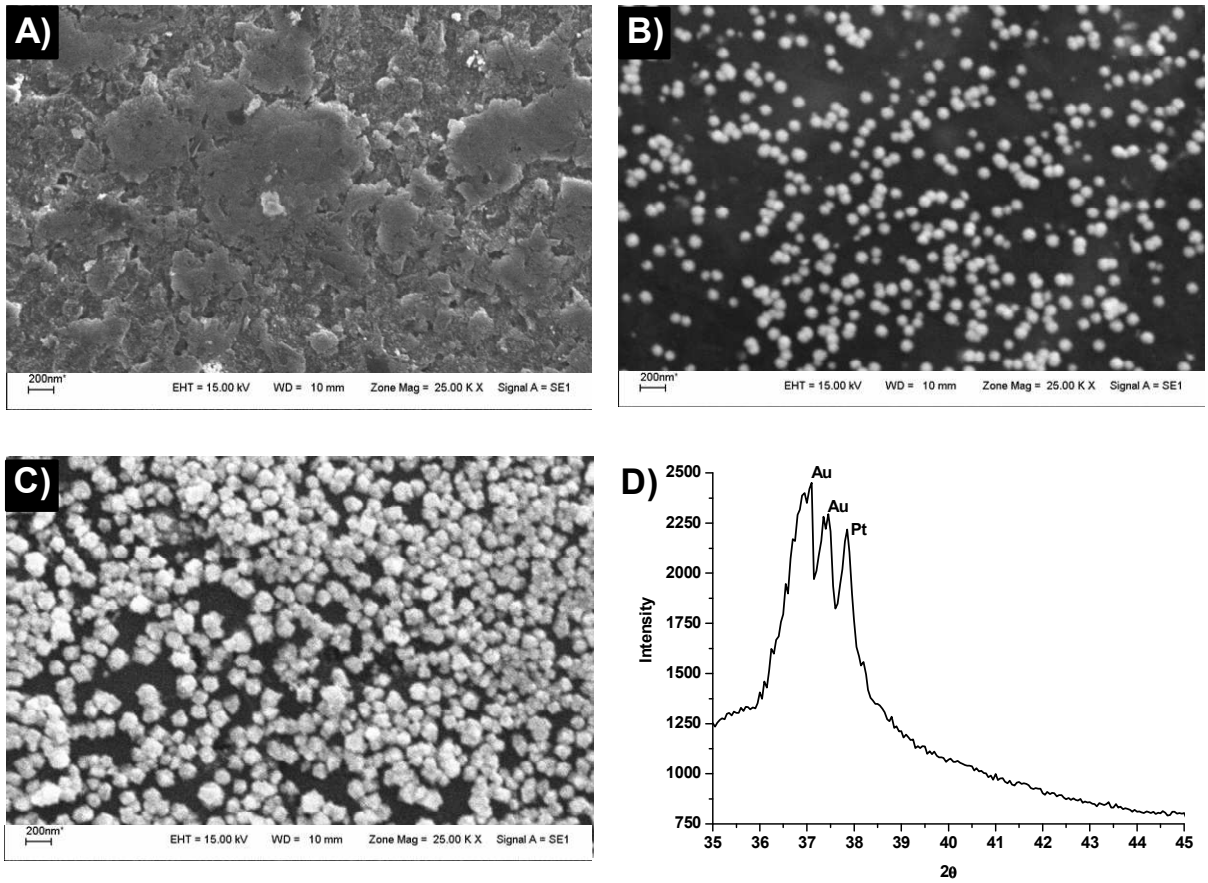


Fig. 2

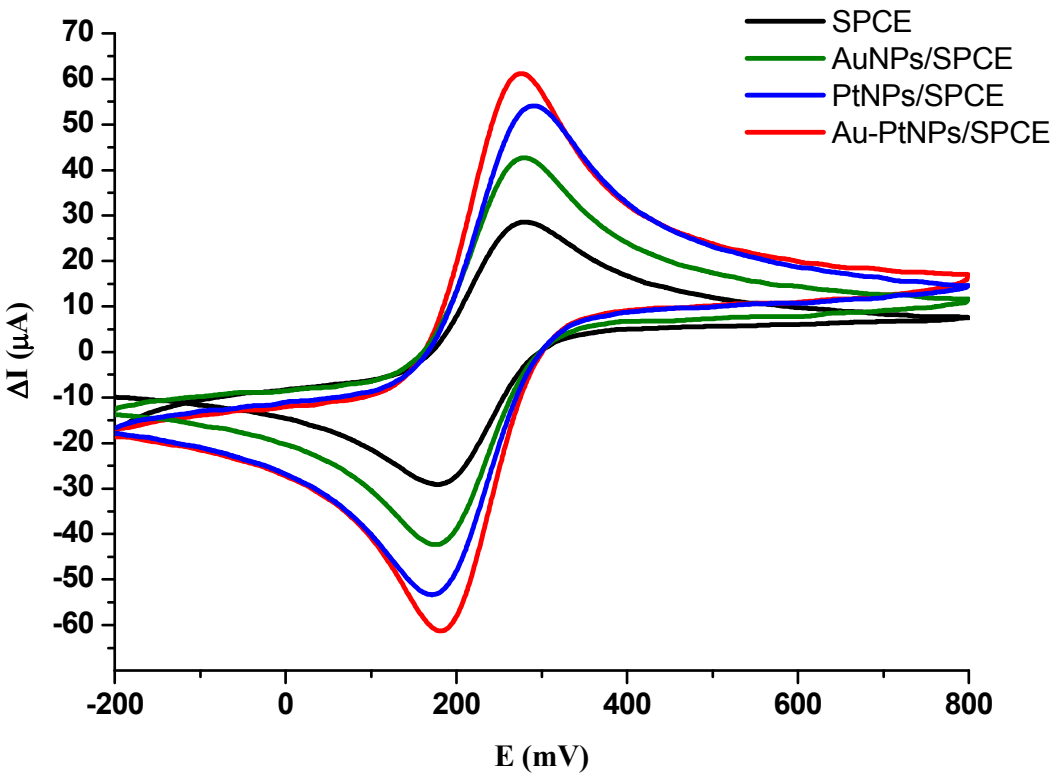


Fig. 3

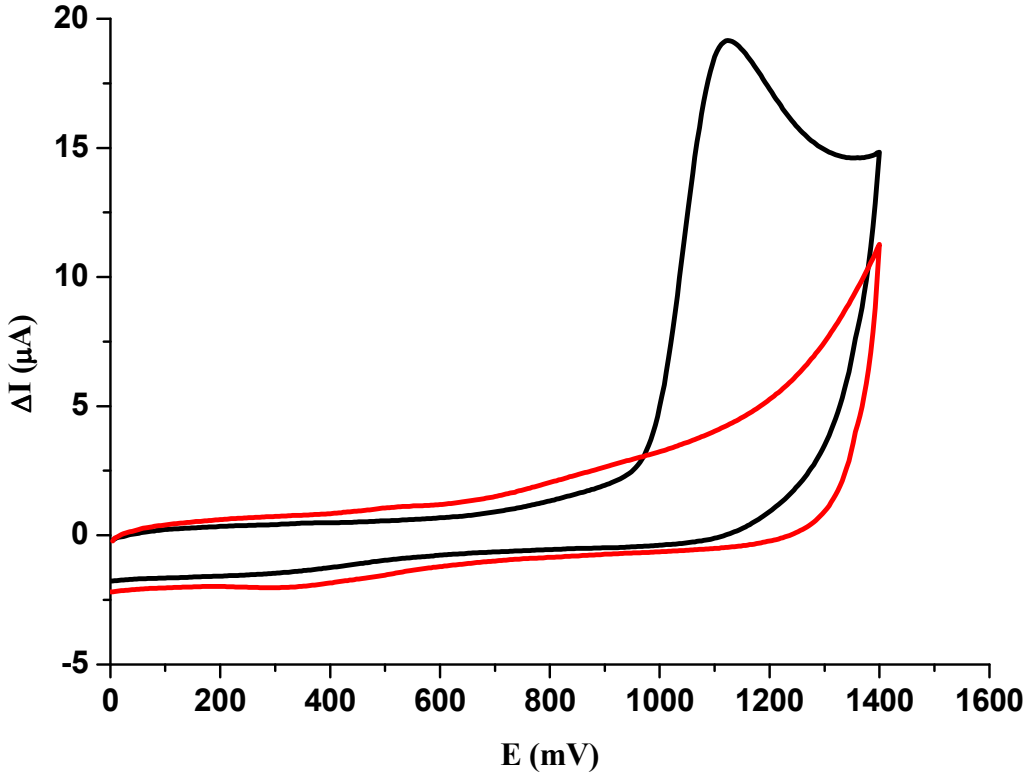


Fig. 4

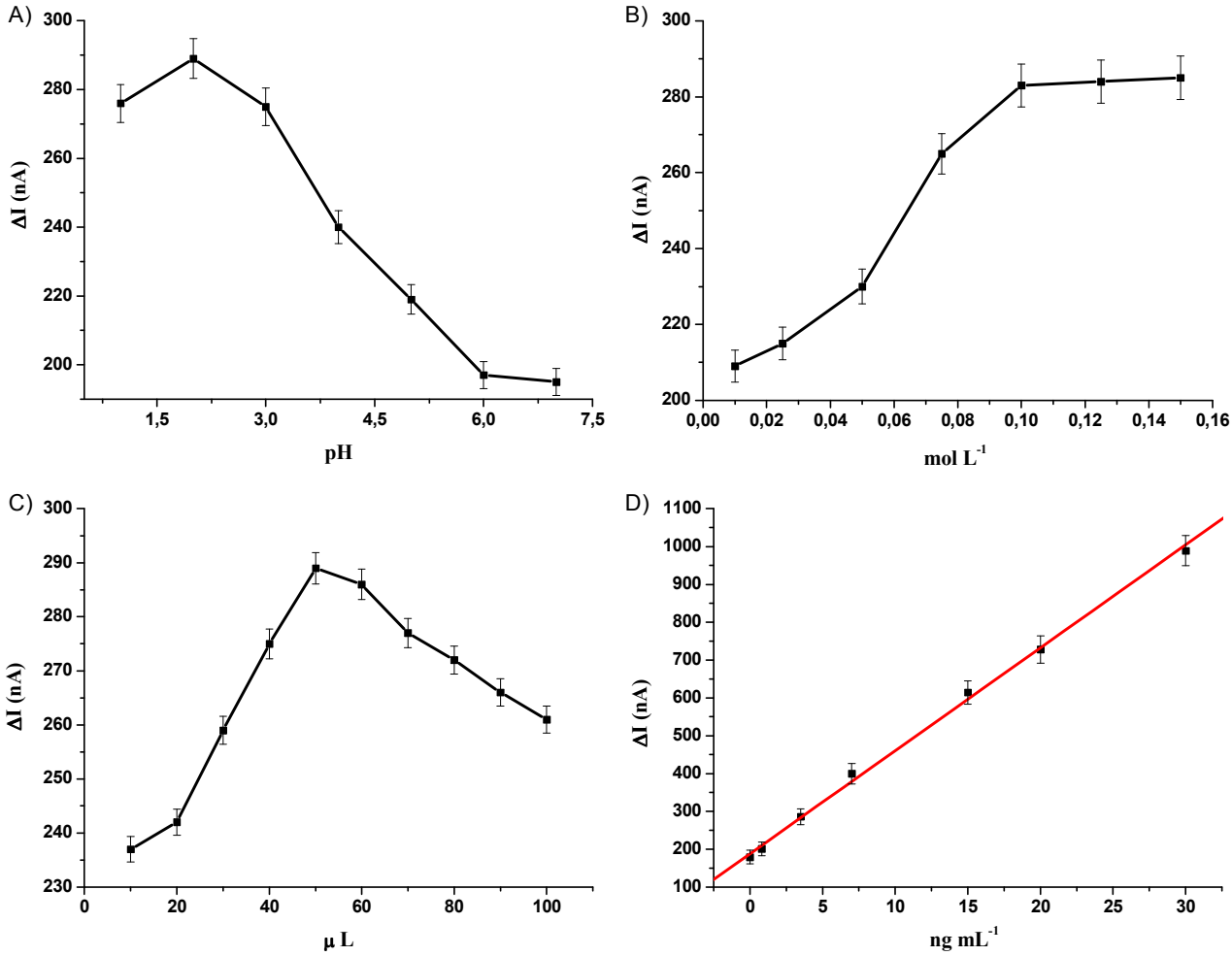


Fig. 5

Table 1. Comparison of the proposed method with previously reported methods for the ZER determination.

Modification	Electrode	Technique	Sample	Linear range (ng mL^{-1})	LOD (pg mL^{-1})
CS-PtCo-Ab/graphene nanosheets[42]	GCE	CV[a]	Urine	0.05-5	13
Na-Mont-TH-HRP-Ab2/nanoporous gold films-Ab1[43]	GCE	A[b]	Liver	0.01-12	3
Graphene sheets-nickel hexacyanoferrate nanocomposites-Ab[44]	GE	A	Liver	0.05-10	6
Anti-ZER/Au-PtNPs (proposed method)	SPCE	SWV[c]	Urine	0.03-30	10

[a] CV: Cyclic voltammetry
[b] A: Amperometry
[c] SWV: Square wave voltammetry

Table 2. Determination of ZER in bovine urine samples by both methods.

Sample[a]	*Specimen 1*	*Specimen 2*	*Specimen 3*	*Specimen 4*	*Specimen 5*	*Specimen Control*
VI[b]	0.21[d] ± 0.01[e]	0.52 ± 0.02	1.12 ± 0.05	7.23 ± 0.22	18.15 ± 0.61	-
EIS[c]	0.22 ± 0.02	0.50 ± 0.02	1.14 ± 0.07	6.99 ± 0.21	18.32 ± 0.55	-

[a] Bovine urine samples
[b] Voltammetric immunosensor
[c] Enzyme immunoassay
[d] ng mL^{-1}
[e] Mean of five determinations ± S.D.