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Nanostructured voltammetric sensor for ultra-trace anabolic drug determination in food safety field

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ABSTRACT

A sensitive electrochemical sensor for clenbuterol (CL) determination in bovine urine samples was designed. This drug was originally developed for the treatment of respiratory pathologies. However, in the livestock industry, CL has been used as growth promoter because it stimulates the muscle growth and lipid degradation. The drug residues accumulated in animal tissues represent a potential risk for the human health. Subsequently, the development of a fast and simple method for the CL quantification employed to detect its abuse in livestock production is an attractive analytical challenge.

The proposed method was based on the use of a screen printed carbon electrode (SPCE) modified with multiwall carbon nanotubes (MWCNTs) in a matrix of chitosan (CH) on gold nanoparticles (AuNPs) previously incorporated by electrodeposition process. The MWCNTs-CH/AuNPs/SPCE was characterized by cyclic voltammetry, X-ray diffraction and scan electron microscopy.

With the aim to carry out the CL determination, a pre-concentration procedure was required. For this purpose, we employed anti-CL antibodies immobilized on magnetic microparticles (MPs) as bioaffinity support to capture the drug present in the sample. This support was exposed to a desorption process. Later, the electrochemical detection by square wave voltammetry (OSWV) was performed. The sensor exhibited excellent performances with a linear response range in concentrations from 0.01 to 6 ng mL^{-1}. The detection and quantification limits obtained were 0.003 and 0.01 ng mL^{-1}, respectively with an analysis time of 19 min and a R.S.D. below 5.8%. As a conclusion, we can claim that the results obtained by the proposed sensor were successful.

1. Introduction

Nowadays, the detection of compounds present in food which could represent a risk to human health has taken great relevance. Among these kinds of substances we can find the family of β-agonists, which includes compounds such as CL. This drug was originally prescribed for the treatment of respiratory pathologies due to its potent bronchodilator action [1,2]. However, because this compound promotes muscle growth and lipid degradation, it is considered an anabolic substance used to achieve higher livestock production in less time [3].

Moreover, due to its long half-life and stability, CL residues could present a potential risk to human health [4,5], and has to be carefully monitored. Several effects caused by the intake of animal products containing CL were documented, among them: numbness of the hands, tremors and muscle pain, nervousness, headache, tachycardia and myocardial necrosis by decreased perfusion [6,7]. Its use in animal production is banned in Argentina as in most countries including the European Union [8].

Due to the effects previously mentioned several analytical methods have been developed for the CL determination in many biological samples, including ultra high-performance liquid chromatography–mass spectrometry (UHPLC–MS) [9], gas chromatography–mass spectrometry (GC–MS) [10], liquid chromatography–mass spectrometry (LC–MS) [11–13], surface-enhanced Raman scattering (SERS) [14], capillary electrophoresis (CE) [15], enzyme-linked immunosorbent assay (ELISA) [16] and immunosensors [17].

Although the CL determination by these methods has been extensively used in different sample matrices, because of their

Abbreviations: CL, clenbuterol; SPCE, screen printed carbon electrode; MWCNTs, multiwall carbon nanotubes; CH, chitosan; AuNPs, gold nanoparticles; MPs, magnetic microparticles; OSWV, square wave voltammetry.

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high selectivity and sensitivity, most of these are associated with several shortcomings, including high cost, complicated sample pre-treatments and long analysis time. Alternatively and since CL contains an electroactive aromatic amino group, the electrochemical detection should be considered. Among the electrochemical techniques, the OSWV combined with the use of SPCE as detection system represents an interesting option. SPCE offers many benefits such as simplicity, versatility, modest cost and minimum sample volume required (20–40 μL drop) [18,19] and the possibility to carry out the electrodeposition of metal NPs as AuNPs onto its surface, providing an increased active surface, high conductivity and electrocatalytic characteristics to improve the detection limit [20,21].

Another strategy that could be performed to achieve an increase on the sensitivity of the system is the incorporation of MWCNTs in a matrix of CH on modified SPCE surface. This nanomaterial is widely used for sensors due to several properties [22,23], which make it extremely attractive for electrochemical detection [24,25]. Their properties include length/diameter aspect ratio which provides a high surface/volume ratio and ability to promote fast electron transfer for a wide range of electroactive species [26,27].

MPs are typically made of a magnetic iron oxide core surrounded by a non-magnetic polymer functionalized with amino groups, which can be modified with biomolecules of interest [28]. These are often used as solid supports for immunoassay reactions. They feature a large binding surface area per volume and hence a large number of analyte molecules are bound within a small volume, allowing a sensitive detection [29].

The aim of this work was to develop an electrochemical method for CL determination in bovine urine samples employing MWCNTs-CH/AuNPs/SPCE as detection system and MPs as bioaffinity support in a previous pre-concentration procedure.

Anti-CL antibodies previously immobilized on MPs allowed the CL pre-concentration through the immune recognition process. Later the immunologically bounded CL was exposed to a desorption procedure for its subsequent detection by OSWV. The obtained current response was directly proportional to the amount of the CL present in bovine urine samples.

The inherent advantages of electrochemical system and the incorporation of nanomaterials combined with the extraordinary selectivity of antibodies employed in a pre-concentration step with MPs as bioaffinity support results in an attractive and efficient analytical tool to be applied in the food safety field.

2. Experimental and methods

2.1. Chemicals and materials

All reagents used were of analytical reagent grade. Rabbit anti-CL antibody (A1, Supporting Information) and samples were supplied by Viviana G. Spotorno from National Institute of Agricultural Technology (INTA) Argentina. Samples were obtained in compliance with the relevant laws and institutional guidelines approved by the authorities of INTA.

The Clenbuterol Quantitative ELISA kit (101225) was used in accordance with the manufacturer's instructions and was purchased from NEOGEN (USA). CL, CH (from crab shells, medium molecular, 85% deacetylated), gold solution (0.01% as $HAuCl_4$) and MWCNTs (diam. = 110–170 nm, length = 5–9 μm, 90%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Glutaraldehyde (25% aqueous solution) was purchased from Merck (Darmstadt, Germany). MPs amino functionalized (53572) were purchased from Fluka (Buchs, Switzerland). All the other employed reagents were of analytical grade and were used without further purification. Aqueous solutions were prepared by using purified water from a Milli-Q system.

2.2. Apparatus

Electrochemical experiments were performed in unstirred solutions by using a BAS 100B/W electrochemical analyzer (Bioanalytical System, West Lafayette, IN), employing positive feedback routine to compensate the ohmic resistance. Cyclic and square wave voltammograms were obtained by employing a SPCE, it was made up of a graphite circular working electrode (Ø = 3 mm). Silver (Ag) and graphite electrodes were used as the pseudo-reference and the auxiliary electrode, respectively. All the potentials in the text are referred to the Ag. All the experiments were conducted at room temperature (25 ± 1 °C).

Absorbance was detected by a Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 general UV/vis spectrophotometer.

The structure and composition of the nanomaterials were characterized by X-ray diffraction (XRD) using a Rigaku D-MAX IIIC diffractometer with copper radiation (ka = 0.154178 nm) and a nickel filter. The morphologies of the MWCNTs and the electrodeposited AuNPs were studied by a LEO 1450VP scanning electron microscope (SEM).

2.3. Immobilization of anti-CL antibodies on MPs

Anti-CL antibodies were immobilized on amino functionalized MPs in an Eppendorf tube. 100 μL of MPs were washed with 1 mL of 0.01 mol L^{-1} PBS pH 7.20 three times. The pellet was suspended in 1 mL of an aqueous solution of 5% (w/w) glutaraldehyde (0.10 mol L^{-1} sodium phosphate buffer, pH 8.0) with continuous mixing for 2 h at room temperature. After three more washes with 0.01 mol L^{-1} PBS pH 7.20, 250 μL of antibody preparation (dilution 1:100 in 0.01 mol L^{-1} PBS pH 7.20) were coupled to the residual aldehyde groups with continuous mixing for 12 h at 4 °C. The preparation containing antibodies immobilized on MPs was finally washed with 0.01 mol L^{-1} PBS pH 7.20 and suspended in 250 μL of the same buffer at 5 °C. Immobilized antibody preparation was stable for at least 1 month.

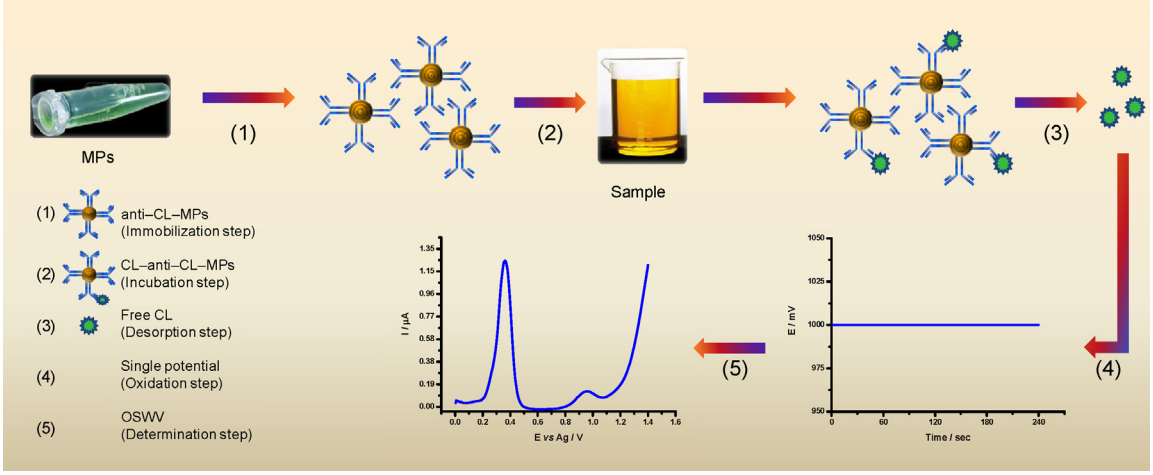
2.4. Preparation of the MWCNTs-CH/AuNPs/SPCE

With the aim to improving the sensitivity and reproducibility of the results, an electrode pre-treatment was carried out before each voltammetric experiment. This treatment generates the oxidation of the graphite impurities and provides a more hydrophilic surface (A2, Supporting Information). After that, the electrodeposition procedure was achieved by immersing the SPCE surface into 500 μL of gold solution ($HAuCl_4$) 0.01% containing 0.1 mol L^{-1} KCl and applying a constant potential value of −200 mV for 60 s. Finally, the modified electrode (AuNPs/SPCE) was rinsed by mechanically stirring at 250 rpm for 30 s with ultrapure water and carefully dried with pure nitrogen gas.

CH solution was obtained by adding 0.1 g of CH to 10 mL (1:1 – ethanol:H_2O) under stirring conditions and maintaining a pH value of 3 through the addition of 0.1 mol L^{-1} HCl solution. The undissolved material was filtered. Later, the pH value was gradually adjusted to pH 7.0 using 0.1 mol L^{-1} NaOH solution. Finally, the obtained filtrate was appropriately diluted to a 0.5% CH solution and stored at 4 °C.

At the same time, to achieve the MWCNTs functionalization, a chemical treatment using a mixture of concentrated acids (3:1 – H_2SO_4:HNO_3) for 12 h was carried out. After this step, the suspension was filtered; the solid was washed with ultrapure water at pH 7.0 and dried at 120 °C for 5 h [30].

With the goal to obtain MWCNTs-CH preparation, 1.2 mg of MWCNTs was dispersed in 1 mL of CH (0.5% in 1:1 – etanol:H_2O solution) with the aid of ultrasonic stirring for 45 min. The



Scheme 1. Schematic representation of the CL quantification procedure in bovine urine samples.

MWCNTs-CH dispersion was kept at 4 °C when not in use and was stable for at least 5 months.

Finally, 5 µL of the MWCNTs-CH previously obtained were dropped on the AuNPs/SPCE working electrode and the solvent was evaporated under the action of an infrared heat lamp.

2.5. Clenbuterol voltammetric determination in bovine urine samples

The procedure for the quantification of CL in six bovine urine samples involves the following stages (Table A1, Supporting Information). Firstly, anti-CL-MPs preparation was conditioned with 1 mL of desorption buffer (0.1 mol L^{-1} glycine pH 2.0) at room temperature for 5 min. After, anti-CL-MPs were rinsed with PBS buffer (0.01 mol L^{-1} pH 7.20) and the unspecific bindings were blocked by a 10 min treatment with 1% of bovine serum albumin (BSA) at 37 °C. Anti-CL-MPs were washed three times in PBS buffer and stored in the same buffer. In the capture step, 100 mL of bovine urine sample (previously diluted in PBS buffer 50 fold) containing CL were incubated with 30 µL of anti-CL-MPs for 10 min at room temperature under shaking conditions. In this step, the CL present in the sample reacted immunologically with anti-CL antibody immobilized on MPs. The obtained CL-anti-CL-MPs complexes were then recuperated using an external removable magnet and washed three times with PBS buffer. Later, 200 µL of desorption buffer were added to the CL-anti-CL-MPs complexes. After 1 min, the CL present in the solution was oxidized by single potential for 4 min at +1000 mV. In this case, the drug generated a dimmer that was adsorbed on the surface of the electrode and then it was determined using OSWV with the following general parameters: step E = 4 mV, S.W. amplitude = 25 mV, S.W. frequency = 15 Hz, samples per point = 256, studied potential range = 0–1400 mV, sensitivity = 1×10^{-5} A V^{-1} (Scheme 1). The obtained current response was directly proportional to the amount of the CL present in the bovine urine samples. The blank solution was prepared in the same way except that instead of the bovine urine sample 100 mL of purified water were employed.

3. Results and discussion

3.1. Characterization of the MWCNTs-CH/AuNPs/SPCE

The SEM images of the SPCE working electrode (A), AuNPs/SPCE (B) and MWCNTs-CH/AuNPs/SPCE (C) are shown in Fig. 1. AuNPs were made on the SPCE surface by electrodepositing at −200 mV from 500 µL of gold solution ($HAuCl_4$) 0.01% in 0.1 mol L^{-1} KNO_3 for 60 s. After the electrodeposition step, AuNPs were uniformly distributed on the working electrode surface. Regarding to the electrodeposition time, we could observe the growth of Au around the already deposited Au-seeds. The formation of new AuNPs decreased with the electrodeposition time. Therefore, the dispersion of the AuNPs size became larger with the increasing electrodeposition time.

The crystalline structure of the MWCNTs-CH/AuNPs/SPCE was characterized by XRD measurement. The XRD pattern is shown in Fig. 1(D). The peaks at 2θ (23.1° and 26.5°) were from graphite and MWCNTs respectively, and the peaks at 2θ (42.6°, 54.3° and 68.9°) resulted from the AuNPs. The average size of the crystalline structure of the deposited AuNPs was calculated according to the Scherrer equation ($t = K \times \lambda / B \times \cos\theta$) and the obtained value was approximately 30 nm.

3.2. Electrochemical characterization of the MWCNTs-CH/AuNPs/SPCE

Cyclic voltammograms of the catechol (Q) system is a convenient and valuable tool to monitor the surface properties of the electrode during different modifying steps. Fig. 2 shows the cyclic voltammograms for six different types of electrodes: bare SPCE (i), CH/SPCE (ii), CH-AuNPs/SPCE (iii), MWCNTs-CH/SPCE (iv), AuNPs/SPCE (v) and MWCNTs-CH/AuNPs/SPCE (vi), which were recorded in PBS buffer (0.01 mol L^{-1} pH 7.20) + 1 mmol L^{-1} Q (scan rate = 50 mV s^{-1}; $T = 25 \pm 1$ °C). Well defined cyclic voltammograms and characteristics of a diffusion-controlled redox process were observed at the bare SPCE surface (i). After the electrode was modified with CH, a marked diminution in the peak current was observed (ii), meaning that a blocking effect which decreases the diffusion of Q toward the electrode surface is present. The curve (iv) illustrates the voltammetric effect of Q on the MWCNTs-CH/SPCE. Due to MWCNTs have a catalytically active surface, they can increase the active surface area of the modified electrode, and therefore, the peak current of Q was greater than the bare SPCE. For CH-AuNPs/SPCE (iii), the peak current was smaller than the MWCNTs-CH/SPCE. These results indicated that AuNPs played a role in the increase of the electroactive surface area (v), and also acted as a conducting wire or electron-conducting tunnel for Q electron transfer, but this increase was significantly affected by the blocking behavior of CH. When the film composed of MWCNTs-CH/AuNPs was used to modify the electrode (vi), the peak current was the highest compared with the other five electrodes owing it to the large surface area of the modified electrode and the synergistic

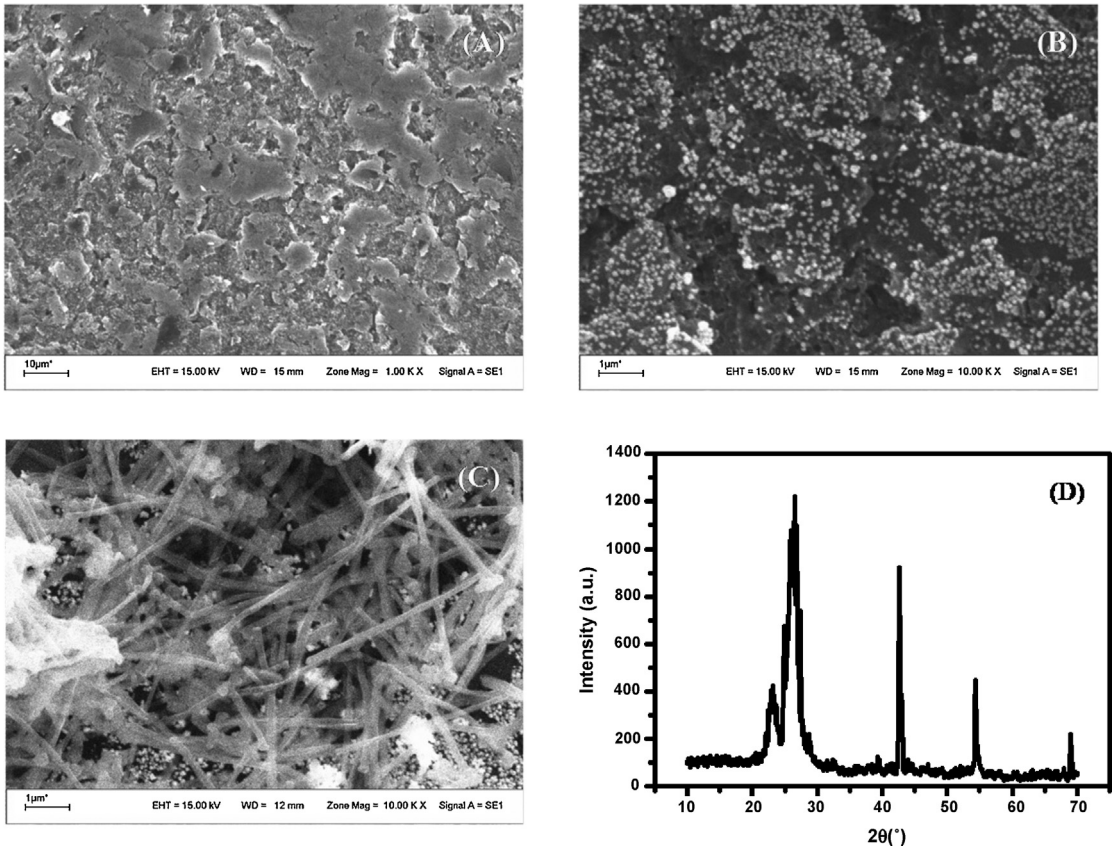


Fig. 1. SEM images of the SPCE working electrode (A), AuNPs/SPCE (B), MWCNTs-CH/AuNPs/SPCE (C) and the corresponding XRD pattern of MWCNTs-CH/AuNPs/SPCE (D).

action of the electrocatalytic activity of AuNPs and MWCNTs. The average value of surface area for MWCNTs-CH/AuNPs/SPCE was 12.35 ($\pm$0.15) $\times$ 10^{-2} cm^2 (n = 6) according to the Randles–Sevick equation [31], that is $I_p = 2.69 \times 10^5\, AD^{1/2}\, n^{3/2}\, \nu^{1/2}\, C$.

3.3. Electrochemical behavior of clenbuterol at the MWCNTs-CH/AuNPs/SPCE

Fig. 3 shows cyclic voltammograms obtained in a 0.1 mol L^{-1} glycine pH 2.0 solution at 50 mV s^{-1} with MWCNTs-CH/AuNPs/SPCE in the absence and presence of 1 mmol L^{-1} CL. As can be seen, the cyclic voltammogram without CL (i) shows essentially no response at the same scanning rate. However, the cyclic voltammograms (ii) and (iii) exhibit typical reduction/oxidation behavior at the electrode surface with CL in the solution. Also, CL at the MWCNTs-CH/AuNPs/SPCE undergoes an ECE process. In the first scan (ii), an anodic peak I_a is observed near ~0.85 V and then the I_a drops sharply in the next scan, suggesting that an adsorbing process occurred on the electrode. The return scan for the first cycle shows a peak at ~0.18 V (II_a). In the second scan, the cyclic voltammogram shows two obvious anodic peaks and one cathodic peak (iii), i.e., I_a (~0.85 V), II_a (~0.18 V) and II_b (~0.29 V), respectively; II_a and II_b currents gradually increase to a stable value, with an increase in cycle time. This mechanism involves the electrochemical generation of a product at high positive potential (~0.85 V) followed by a chemical reaction, whose product exhibits a quasi-reversible couple at ~0.25 V. Additionally, when a new modified electrode was scanned between a range of 0 and 0.6 V, which does not reach the oxidation potential of CL (~0.85 V), the reduction peak on the reverse scan and the subsequent reoxidation peak on the forward scan were absent (not shown). This fact suggested that the quasi-reversible couple is due to a product formed at higher potentials. The nitrogen sp^2 hybridization, which results in CL dimmer formation through an azo bond, was the proposed mechanism for explaining the obtained results (Scheme 2) [32].

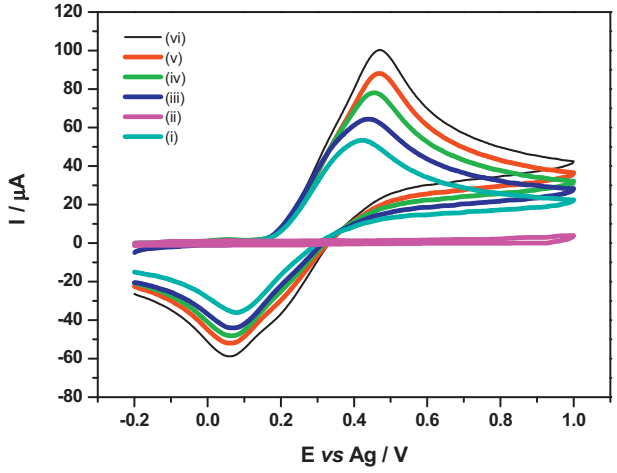


Fig. 2. Cyclic voltammograms obtained in 0.01 mol L^{-1} PBS pH 7.20 in the presence of 1.0 mmol L^{-1} Q at 50 mV s^{-1} for bare SPCE (i), CH/SPCE (ii), CH-AuNPs/SPCE (iii), MWCNTs-CH/SPCE (iv), AuNPs/SPCE (v) and MWCNTs-CH/AuNPs/SPCE (vi), respectively.

3.4. Election of the electroanalytical technique

The quantification step for this reaction can be monitored measuring the CL response, using MWCNTs-CH/AuNPs/SPCE as a working electrode. There are two forms to measure the CL response: (i) measure the oxidation peak at high potentials or (ii) the quasi-reversible couple at low potentials. The election depends

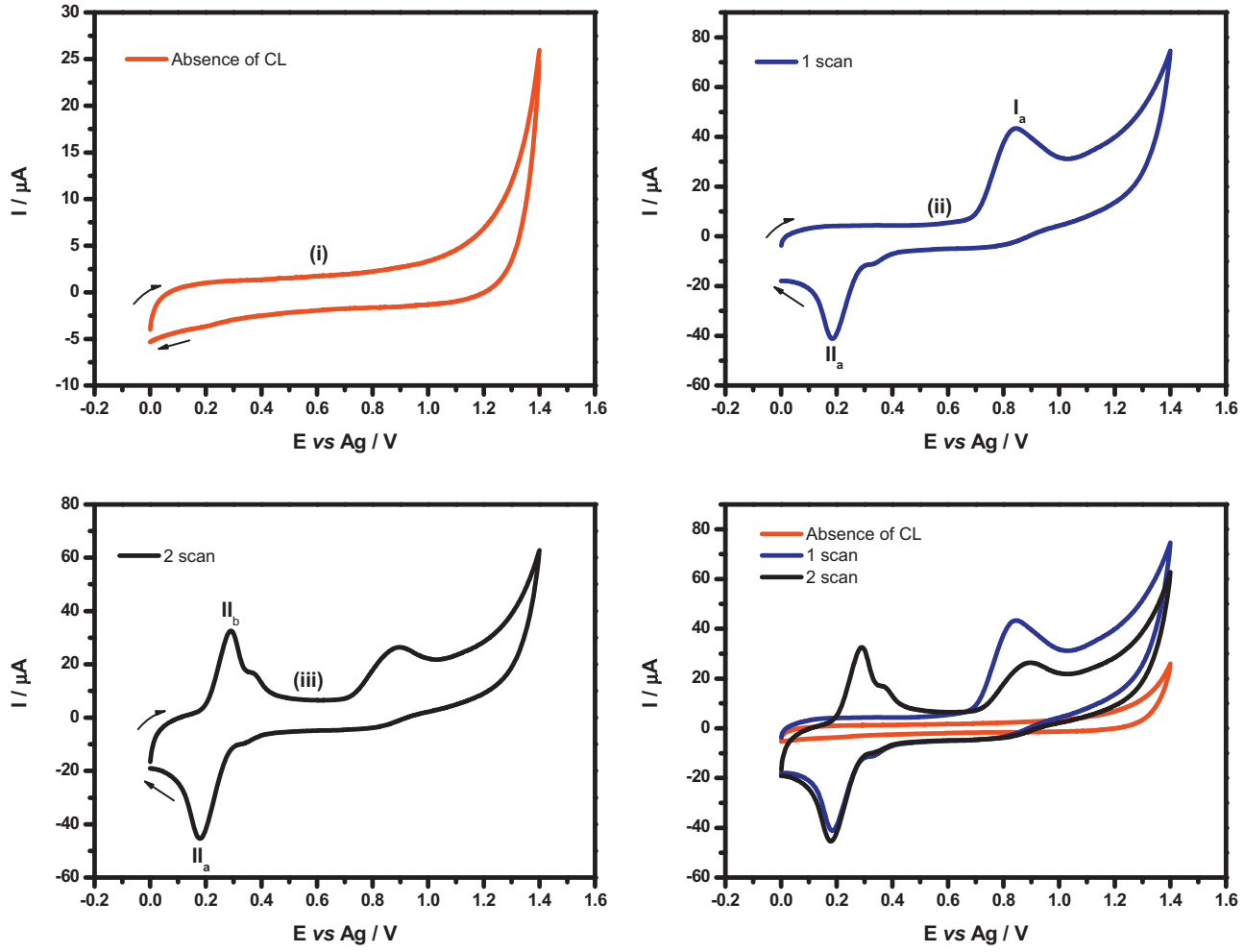
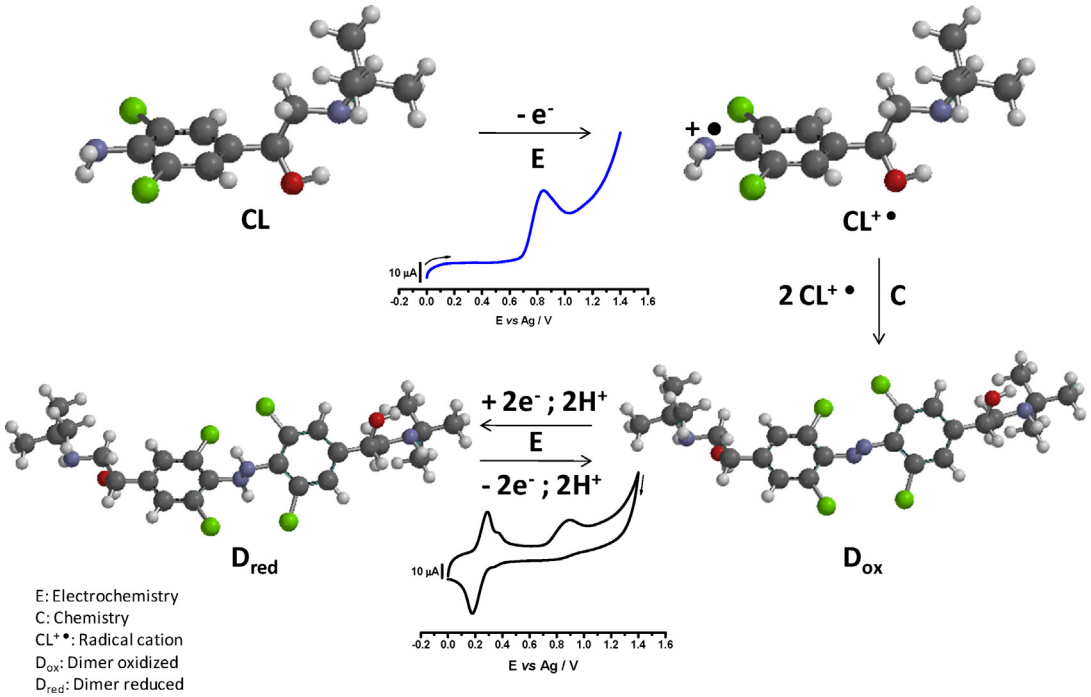


Fig. 3. Cyclic voltammograms obtained in 0.1 mol L^{-1} glycine pH 2.0 in the absence and presence of 1 mmol L^{-1} CL at 50 mV s^{-1} with MWCNTs-CH/AuNPs/SPCE.



Scheme 2. Electrochemical behavior of CL at the MWCNTs-CH/AuNPs/SPCE.

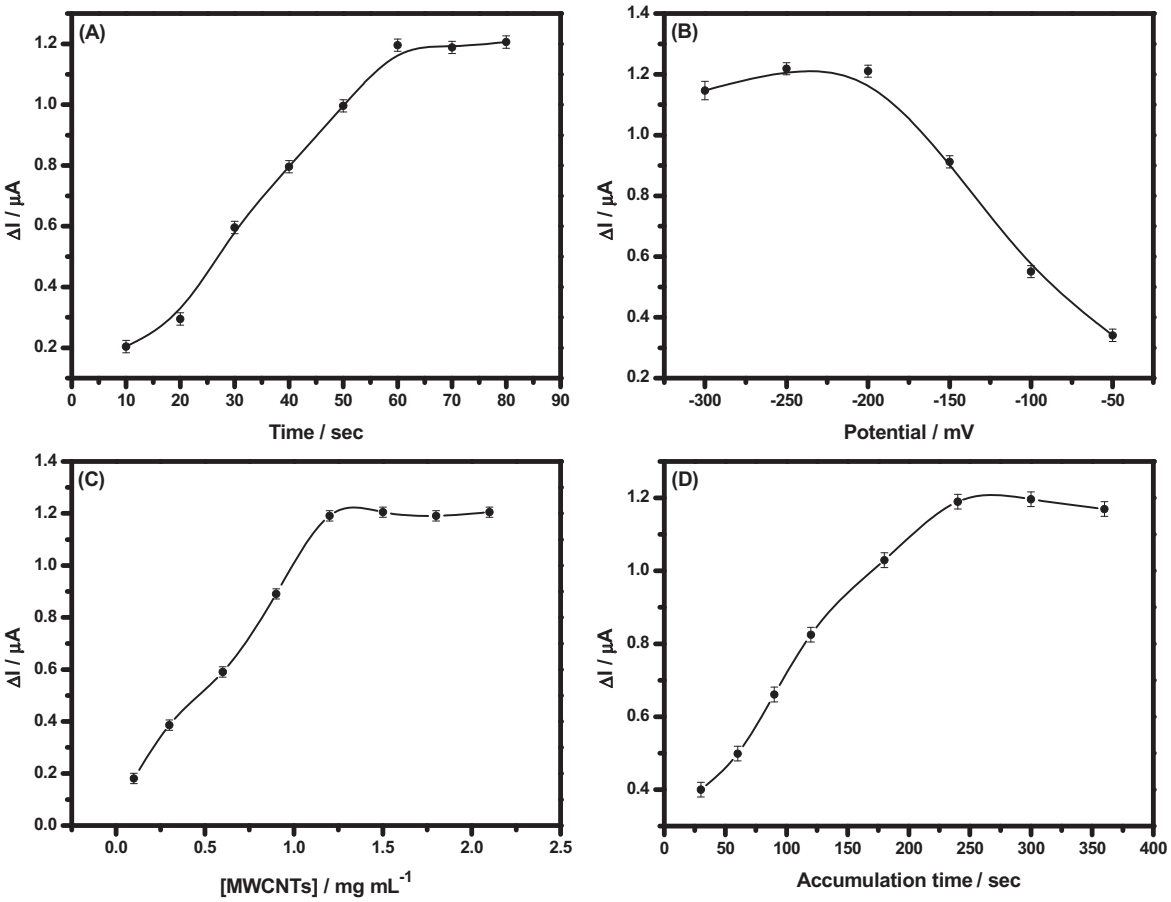


Fig. 4. Study of variables. Electrodeposition time (*Et*) (A), electrodeposition potential (*Ep*) (B), MWCNTs concentration (C) and accumulation time (D), employing a standard of 5.0 ng mL^{-1}.

on the electroanalytical technique to utilize. For OSWV, when the system is reversible or quasi-reversible, the reverse current is very significant, so the difference between currents is greater than the techniques which use either the forward or reverse currents. A greater advantage of OSWV is the possibility to see during only one scan if the electron transfer reaction is reversible or not. Other advantages of OSWV are the great speed of analysis, the low consumption of the electroactive species in relation with differential pulse voltammetry (DPV), and the reduction of the problems with the soiling of the electrode surface [31]. Taken these into account, we can claim the measure of the quasi-reversible couple at ~0.25 V appears to be more sensitive than measure of the direct electrochemical oxidation of CL at ~0.85 V, using OSWV as electroanalytical technique. Therefore, this indirect electrochemical method was suitable for the determination of CL after its pre-concentration and subsequently dimer formation.

3.5. Study of variables

In order to perform the CL voltammetric determinations in bovine urine samples, many variables that affect the electrochemical response and therefore the obtained results must be analyzed. The fixed electrochemical parameters to carry out the optimization of these relevant variables were: step E = 0.010 V, S.W. amplitude = 0.050 V, S.W. frequency = 10 Hz, samples per point = 256, studied potential range = 0–1.40 V, sensitivity = 1×10^{-5} A V^{-1} using MWCNTs-CH/AuNPs/SPCE as working electrode. Regarding to the CL concentration, a standard solution of 5 ng mL^{-1} was employed in all studies.

3.5.1. Electrodeposition time (Et) and potential (Ep)

The time and potential employed for the AuNPs electrodeposition procedure are relevant parameters which affect the sensitivity of the technique, due to the AuNPs provided an increased surface area, high conductivity and electrocatalytic characteristics to improve the detection limit. For these reasons the *Et* was evaluated in a range of 10–80 s. The rate of response increased linearly if the *Et* rises from 10 to 60 s, insignificant differences were observed when *Et* was greater than 60 s (Fig. 4 (A)). So, an *Et* of 60 s was selected as optimum.

Regarding to the *Ep*, the time employed was 60 s and the working electrode potential was varied between −50 and −300 mV. The current increased rapidly by increasing the potential up to a value of −200 mV, and then remained constant between −200 and −300 mV (Fig. 4(B)). Therefore, an *Ep* of −200 mV was selected as optimum. The values used throughout the experiment were 60 s and −200 mV for *Et* and *Ep*, respectively.

3.5.2. MWCNTs concentration

The MWCNTs concentration employed for the modification of electrode surface was also optimized. This study was carried out in the range of 0.1–2.1 mg mL^{-1}. An important increase of the signal was observed between 0.1 and 1.2 mg mL^{-1}. However, at higher concentrations insignificant differences were obtained (Fig. 4(C)).

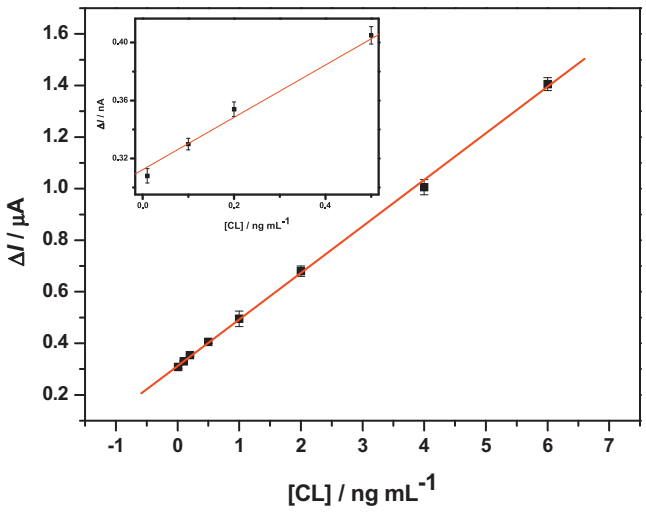


Fig. 5. Calibration curve for CL quantitative determination by OSWV using MWCNTs-CH/AuNPs/SPCE. Voltammetric conditions: 0.1 mol L^{-1} glycine pH 2.0, step E = 0.010 V, S.W. amplitude = 0.050 V, S.W. frequency = 10 Hz, studied potential range = 0–1.40 V.

Table 1
Determination of clenbuterol in bovine urine samples by both methods.

Samples[a]	VS[b]	EIA[c]
BU 1[d]	0.175 ± 0.01[e]	0.168 ± 0.02
BU 2	0.561 ± 0.03	0.511 ± 0.04
BU 3	2.779 ± 0.05	2.8 ± 0.1
BU 4	1.315 ± 0.04	1.389 ± 0.09
BU 5	0.751 ± 0.03	0.790 ± 0.05
BU 6	0.155 ± 0.01	0.145 ± 0.02

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

Then 1.2 mg mL^{-1} of MWCNTs was used for the modification process.

3.5.3. Accumulation time

The accumulation time employed for the oxidation of CL was also optimized. As shown in Section 3.4, the measurement of the quasi-reversible couple at ~0.25 V offers more sensitivity than the measurement of the direct electrochemical oxidation of CL at ~0.85 V. Therefore, the potential was fixed to +1.0 V to assure the CL oxidation, and then the accumulation time was changed. Fig. 4(D) shows a linear accumulation response up to 240 s, after this value a saturation process was observed. Therefore, 240 s were used for all experiments.

In addition, other variables such as volume of MPs, concentration and pH of the desorption buffer were studied (A3 and A4, Figures A1, A2 and A3, Supporting Information).

3.6. Analytical performance

Linearity and range of the developed method were studied by analyzing different concentrations ($n = 5$) of standard solution containing 0.001–10 ng mL^{-1} of CL for an 100 mL of bovine urine sample (previously diluted in 0.01 mol L^{-1} PBS pH 7.20 50 fold). A linear relation was observed between the concentration range of 0.01–6 ng mL^{-1}. The calibration graph was described according to the following equation: ΔI (μA) = 0.3124 (±0.0026) + 0.1805 (±0.0035) [CL] (ng mL^{-1}) with a correlation coefficient of 0.998, where ΔI is the difference between current of the blank and sample (Fig. 5).

The detection (LOD) and quantification (LOQ) limits were determined according to the IUPAC recommendations [33], achieving values of 0.003 and 0.01 ng mL^{-1}, respectively.

The repeatability of the CL determinations using the MWCNTs-CH/AuNPs/SPCE was carried out in 0.1 mol L^{-1} glycine at pH 2.0 measuring the current (ΔI) by OSWV in the presence of 5 ng mL^{-1} CL. The relative standard deviation (R.S.D.) for $n = 5$ was 2.2%. Additionally, we evaluated the reproducibility of sensor once a week during one month, under the same conditions described above, showing a R.S.D. of 4.1%.

The stability of MWCNTs-CH/AuNPs/SPCE was evaluated by 45 voltammetric determinations of 5 ng mL^{-1} and no significant change was observed in the voltammetric responses.

In order to evaluate the analytical applicability, the proposed sensor was applied to CL voltammetric determination in six bovine urine samples under the conditions previously described. The CL 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 1).

Table 2 shows previously published articles for CL determination in different samples such as serum, liver, meat, and feed, among others. The new method has significant advantages over the previously reported. One of these is that the present method is based on the use of OSWV as detection technique which offers sensitive determinations in short analysis time. Other advantages are the employment of SPCE modified with a combination of nanomaterials

Table 2
Experimental conditions and analytical parameters for clenbuterol determination of the sensors reported in the literature.

Modified electrodes	Method	Matrix	Linear range (ng mL^{-1})	LOD (ng mL^{-1})
MWCNT-Nafion Nanocomposite [34] (GCE)[a]	DPV[e]	Urine	0.276–276	0.14
Carbon Nanotube [26] (GCE)	DPV	Feed	0.8–1000	0.32
Polyelectrolyte/Nano Gold Particle Layer-by-Layer Assembly [35] (GCE)	Amperometric	Meat	0.5–10	0.20
Chitosan/Nafion/Nano-Silver/Poly Quercetin [36] (CPE)[b]	Amperometric	Liver	83.16–13860	2.772
Graphene Oxide–Ag Composites [37] (GCE)	DPV	Urine	0.01–10	0.007
Multiwalled Carbon Nanotube4-tert-butyl calix[6]arene Composite [38] (CPE)	DPAdSV[f]	Urine and Serum	5.47–13107	0.38
Gold Nanoparticles [39] (SPCE)[c]	Amperometric	Hair	0.027–800	0.008
Platinum Nanoparticles [40] (GE)[d]	DPV	Urine	27.72–221.76	12.18
MWCNTs-CH/AuNPs (SPCE)	OSWV[g]	Urine	0.01–6	0.003

[a] Glassy Carbon Electrode.
[b] Carbon Paste Electrode.
[c] Screen-Printed Carbon Electrode.
[d] Gold Electrode.
[e] Differential Pulse Voltammetry.
[f] Differential Pulse Adsorptive Stripping Voltammetry.
[g] Osteryoung Square Wave Voltammetry.

(MWCNTs-CH/AuNP) which provide increased sensitivity to the system and a selective pre-concentration step with anti-CL antibodies bounded on the MPs surface. In addition, the achieved LOD is lower than that obtained by the methods recently reported.

To conclude, in the best of our knowledge, no study involving MPs with OSWV using MWCNTs-CH/AuNP/SPCE as working electrode for clenbuterol determination at ultra-trace levels has been reported.

4. Conclusions

The goal of this work was to develop a method that could be easily employed for the adequate CL determination in bovine urine samples, due to the abuse of this drug as growth promoter represents a risk for meat consumers.

The proposed sensor combines the use of SPCE with electrodeposited AuNPs and the incorporation of MWCNTs. This combination of nanomaterials enabled us to achieve an increased active area, an improved sensibility and a low limit of detection. Another relevant feature of our system is the selectivity which was provided by employing anti-CL-MPs to capture CL present in the samples. These particles greatly improve the performance of the immunological reaction and have shown to be one of the most powerful and effective platforms for immobilizing bioactive materials. In addition, allowed us to perform the analysis at short time, requiring only 19 min, much less than the time normally used with conventional enzyme immunoassay. These features make of it an adequate and significant tool for CL determination in food safety field.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2013.07.117.

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