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Speciation of chromium using chronoamperometric biosensors based on screen-printed electrodes

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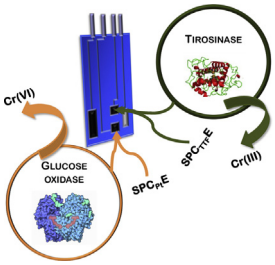
HIGHLIGHTS

- Chronoamperometric determination of Cr(III) on tyrosinase based biosensors using SPCEs.
- Chronoamperometric determination of Cr(VI) on GOx based biosensors using SPCEs.
- High degree of sensitivity and selectivity in the analysis of both chromium species.
- Bipotentiostatic chronoamperometric determination of both chromium species in the same sample.

GRAPHICAL ABSTRACT



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ABSTRACT

Chronoamperometric assays based on tyrosinase and glucose oxidase (GOx) inactivation have been developed for the monitoring of Cr(III) and Cr(VI). Tyrosinase was immobilized by crosslinking on screen-printed carbon electrodes (SPCEs) containing tetrathiafulvalene (TTF) as electron transfer mediator. The tyrosinase/SPC$_{TTF}$E response to pyrocatechol is inhibited by Cr(III). This process, that is not affected by Cr(VI), allows the determination of Cr(III) with a capability of detection of 2.0 ± 0.2 μM and a reproducibility of 5.5%. GOx modified screen-printed carbon platinised electrodes (SPC$_{Pt}$Es) were developed for the selective determination of Cr(VI) using ferricyanide as redox mediator. The biosensor was able to discriminate two different oxidation states of chromium being able to reject Cr(III) and to detect the toxic species Cr(VI). Chronoamperometric response of the biosensor towards glucose decreases with the presence of Cr(VI), with a capability of detection of 90.5 ± 7.6 nM and a reproducibility of 6.2%. A bipotentiostatic chronoamperometric biosensor was finally developed using a tyrosinase/SPC$_{TTF}$E and a GOx/SPC$_{Pt}$E connected in array mode for the simultaneous determination of Cr(III) and Cr(VI) in spiked tap water and in waste water from a tannery factory samples.

1. Introduction

Interest in chemical speciation has recently increased in many fields including biological and environmental sciences. In this way, toxic interactions of heavy metals as well as their bioavailability can be related to the concentration level of a specific chemical form, rather than to the total element content. In the case of chromium, the interest in speciation arises from the difference in

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toxicity of the two most common oxidation states of this element: Cr(III) and Cr(VI). While Cr(III) is an essential species for the proper functioning of living organisms, Cr(VI) presents toxic effects on biological systems being classified as an important carcinogen by the International Agency for Research on Cancer [1]. Moreover, occupational exposure to hexavalent chromium compounds leads to a variety of clinical problems namely, perforation of the nasal septum, asthma, bronchitis, pneumonitis, skin allergies or dermatitis, among others [2].

Chromium is commonly used in numerous industrial processes including pigment production, electroplating or tanning. Thus, large quantities of chromium compounds can be released in liquid, solid and gaseous wastes into the environment, where can ultimately have significant adverse ecological effects [3]. Therefore, the development of accurate and sensitive methods to monitor both species of chromium is necessary in order to determine its fate in the environment and its risk to human health.

Speciation procedures of chromium are generally achieved by combining tedious steps of sample preparation with different analytical techniques. In this way, atomic absorption spectrometry (AAS) has been applied to the analysis of Cr(III) and Cr(VI) using previous extraction procedures including, liquid microextraction [4–10], solid phase extraction [11–16] and cloud point extraction (CPE) [17–22]. AAS has also been used in the speciation of chromium after tedious coprecipitation [23,24], preconcentration [25–29] and adsorption [30,31] stages. Chromatographic techniques such as HPLC have been also applied in the simultaneous determination of both chromium species using previous preparation steps [32,33] and also hyphenated with inductively coupled plasma mass spectrometry (ICP-MS) [6,9,11,23,31]. These analytical systems show important drawbacks including complex procedures, high cost of the necessary instrumentation and long analysis time. Therefore, these methods show a low applicability in routine determinations.

Electrochemical techniques present advantages such as high sensitivity, relative simplicity, low cost and automatic on-line and portable options. Thus, Cr(III) and Cr(VI) have been simultaneously determined by means of differential pulse polarography [34] and, especially, adsorptive stripping voltammetric techniques [35,36]. Most of these methods are based on the use of a hanging mercury drop electrode (HMDE) as working electrode [37–44]. Despite the good results obtained with mercury electrodes, the use of HMDE is nowadays being reduced due to its environmental pollution problems. In this way, a promising tool for the electrochemical detection and speciation of heavy metals is the use of assays based on the inhibition of enzymes [45]. Cr(III) has been successfully determined using an amperometric biosensor based on the inhibition of the enzyme tyrosinase using a glassy carbon electrode [46]. Cr(VI) has been also analyzed using amperometric biosensors based on the inhibition of glucose oxidase (GOx) immobilized on a Pt electrode [47] and of cytochrome c3 on a glassy carbon electrode [48]. This inhibitory effect of metal ions on enzyme activity is due to their binding to thiol groups of protein amino acids habitually forming the active centre of the enzyme [47,49,50]. The possibilities of amperometric biosensors can be increased by using screen-printed electrodes (SPEs) due to their disposable character and their particular applicability for in situ determinations [51].

In this work, chronoamperometric biosensors for the selective determination of Cr(III) and Cr(VI) have been developed using two different enzyme modified SPEs. The constructed biosensors allow the selective determination of a chromium species in the presence of the other.

In addition, a novel enzyme-based bipotentiostatic amperometric biosensor has been developed. The electrode system consists of two screen-printed working electrodes connected in array mode. Tyrosinase enzyme was immobilized on one working electrode and GOx on the other one, in order to perform the simultaneous determination of Cr(III) and Cr(VI) in the same sample.

2. Experimental

2.1. Reagents

SPEs were fabricated using different commercial inks. Electrodag 418 Ag ink and Electrodag 6037 SS Ag/AgCl ink were purchased from Acheson Colloiden (Scheemda, The Netherlands). C10903P14 carbon ink, C2050804D9 platinised carbon ink and D2071120D1 dielectric ink were obtained from Gwent Electronic Materials (Torfaen, UK).

All the reagents used were of analytical grade and Milli Q water (Millipore, Bedford, USA) was employed for preparing all solutions. Electrochemical measurements were carried out using a 100 mM phosphate (KH_2PO_4, Sigma–Aldrich, Steinheim, Germany) and 50 mM KCl (Merck, Darmstadt, Germany) solution as supporting electrolyte. From this, solutions of different pH values were prepared by addition of a 2 M sodium hydroxide solution (J.T. Baker, Deventer, The Netherlands) and 500 mM orto-phosphoric acid solution (Panreac, Barcelona, Spain).

Stock standard solutions of Cr(VI) were daily obtained by diluting the adequate volume of a 1000 ppm chromate standard solution (Merck, Darmstadt, Germany) in phosphate buffer. Cr(III) solutions were daily prepared from $Cr(NO_3)_3{\cdot}9H_2O$ (Panreac, Barcelona, Spain) in phosphate buffer.

Potassium ferricyanide was purchased from Merck (Darmstadt, Germany). D(+)-glucose monohydrate and pyrocatechol were obtained from Fluka (Stenheim, Germany).

GOx (EC 1.1.3.4, type X-S: from Aspergillus niger., 129.9 units mg^{-1}) and tyrosinase (EC 1.14.18.1, from mushroom, 3130 units mg^{-1}) enzymes were obtained from Sigma–Aldrich (Steinheim, Germany). Bovine serum albumin (BSA) and glutaraldehyde (GA), used in the immobilization of the enzymes, were both purchased from Sigma–Aldrich (Steinheim, Germany). BSA, tyrosinase and GOx solutions were prepared in 50 mM phosphate pH 6 buffer solution. GA solutions were prepared in high purity water.

Tetrathiafulvalene (TTF) was provided by Sigma–Aldrich (Steinheim, Germany).

2.2. Apparatus

SPEs were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke.

Electrochemical measurements were made using a PalmSens Interface with PalmSensPC software (Palm Instruments BV, Houten, The Netherlands). The pH of solutions was measured with a Hanna instruments HI 221 pH meter (USA).

2.3. Biosensor manufacturing

Two different electrode systems, including one or two working electrodes, have been home constructed according to previously procedures described in [52].

The optimization of experimental variables in the determination of Cr(III) and Cr(VI) were separately made using a single working electrode system. This device consists of three electrodes: a Ag/AgCl reference electrode, a carbon counter electrode and a working electrode (15.9 mm^2). For the determination of Cr(III) the carbon working electrode was modified with TTF ($SPC_{TTF}Es$). In this case, TTF in a 3% (w/w) proportion was incorporated directly into the carbon ink for printing the working electrode. For determination of Cr(VI), the working electrode was constructed by screen-printing platinised carbon paste ($SPC_{Pt}Es$).

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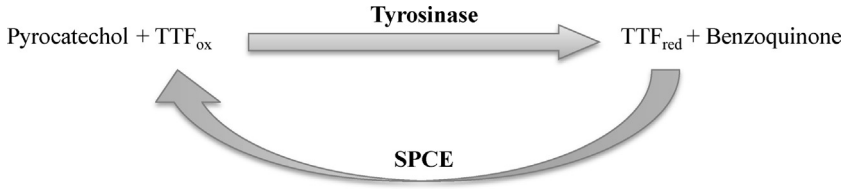

Scheme 1. Enzymatic mechanism of tyrosinase/SPC$_{TTF}$Es biosensors.

In the simultaneous determination of both species of chromium an electrode system based on two different working electrodes (4 mm^2) connected in array mode was used. One working electrode was constructed by screen-printing carbon ink modified with TTF (3% w/w) and the other was obtained from carbon platinised ink.

Tyrosinase enzyme was immobilized by the following cross-linking procedure: a volume of 5 μL of a mixture containing GA (2.5%, v/v), BSA (3%, w/v) and tyrosinase (51.6 un μL^{-1}) in the volume ratio 1:1:3 was deposited on the surface of the SPC$_{TTF}$Es (tyrosinase/SPC$_{TTF}$Es). Then, it was left to react for 90 min at 4 °C.

The cross-linking immobilization of GOx enzyme was carried out by depositing 5 μL of a mixture containing GA (2.5%, v/v), BSA (3%, w/v) and GOx (0.5%, w/v) in the volume ratio 1:1:3 on the surface of the SPC$_{Pt}$E (GOx/SPC$_{Pt}$Es). Then, it was left to react for 90 min at 4 °C.

2.4. Chronoamperometric determination of Cr(III)

Cr(III) determination was carried out by chronoamperometry using tyrosinase/SPC$_{TTF}$Es. All measurements were made at room temperature. The developed biosensors were placed in an electrochemical cell containing 5 mL of 100 mM phosphate and 100 mM KCl solution at pH 5. The working electrode operated at +250 mV vs a screen-printed Ag/AgCl reference electrode under constant stirring. Once the baseline was established, a volume of 150 μL of a 100 mM pyrocatechol solution was added to the measuring cell and a large oxidation signal was observed. Next, when a steady-state response was again observed, additions of 100 μL of a 0.1 mM Cr(III) solution were subsequently made reaching a plateau each time.

2.5. Chronoamperometric determination of Cr(VI)

Analogously, chronoamperometric analysis of Cr(VI) was performed using GOx/SPC$_{Pt}$Es. All measurements were made at room temperature. The developed biosensors were placed in an electrochemical cell containing 5 mL of 100 mM phosphate (pH 4) solution and 1 mM potassium ferricyanide as mediator. The working electrode operated at +200 mV vs a screen-printed Ag/AgCl reference electrode under constant stirring. Once the baseline was established, a volume of 400 μL of a 500 mM glucose solution was added to the measuring cell and a large oxidation signal was observed. Then, when a steady-state response was observed, fixed portions of the 100 μL of a 5 μM Cr(VI) solution were added consequently reaching a plateau each time.

2.6. Simultaneous determination of both species of Chromium by chronoamperometry

The simultaneous determination of Cr(III) and Cr(VI) was carried by chronoamperometry using a tyrosinase/SPC$_{TTF}$E and a GOx/SPC$_{Pt}$E connected in array mode. All measurements were made at room temperature under constant stirring in a cell containing 5 mL of a 100 mM phosphate (pH 4), 50 mM KCl and 1 mM ferricyanide solution. Successive additions of 100 μL of a 0.1 mM Cr(III) and Cr(VI) solution were made.

3. Result and discussion

The main goal of this paper is the development of a sensitive method for the simultaneous determination of Cr(III) and Cr(VI). This point implies the use of a bipotentiostaic amperometric system based on two different working electrodes namely, tyrosinase/SPC$_{TTF}$E and a GOx/SPC$_{Pt}$E. Previous to the simultaneous analysis of Cr(III) and Cr(VI), an optimization process of the experimental parameters has been separately carried out for both chromium species.

3.1. Tyrosinase/SPC$_{TTF}$Es biosensors for the determination of Cr(III)

The selective chronoamperometric determination of Cr(III) is possible using biosensors based on tyrosinase modified screen-printed carbon electrodes (SPCEs). The use of a redox mediator may improve the analytical performance of this biosensor by decreasing the working potential, which significantly reduces the possibility of interference effects. Different mediators were examined founding that good analytical results in terms of sensitivity and selectivity, were obtained when using TTF. Moreover, the direct incorporation of this compound to the screen-printing ink simplifies the manufacture of the biosensor. Thus, tyrosinase enzyme was immobilized on SPCEs modified with TTF. This immobilization was performed following the cross-linking procedure described above.

The developed tyrosinase/SPC$_{TTF}$E biosensor produced a chronoamperometric signal for pyrocatechol related to the mediator oxidation, according to Scheme 1. Fig. 1 shows the time-dependent response of the tyrosinase/SPC$_{TTF}$E biosensor. The response increase observed at around 560 s is due to the addition of pyrocatechol. The decrease in the amperometric response due to the successive additions of Cr(III) into the pyrocatechol solution, clearly indicates that this species inhibits the activity of tyrosinase

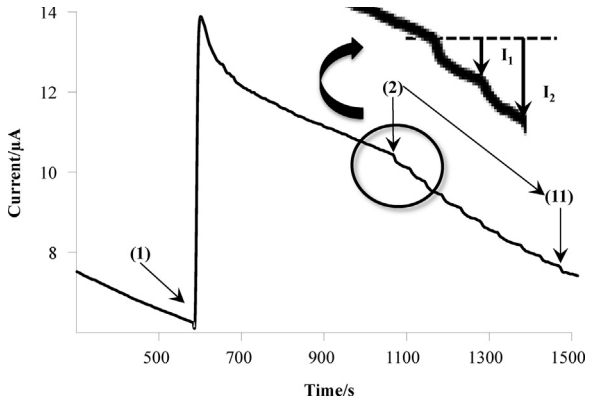

Fig. 1. Chronoamperometry experiments using a tyrosinase/SPC$_{TTF}$E biosensor in 100 mM phosphate and 100 mM KCl solution (pH 5). (1) Addition of pyrocatechol (2.8 mM); (2–11) additions of 100 μL of 0.1 mM Cr(III) solution. ΔI_1, decrease in the current after first addition of Cr(III); ΔI_2, decrease in the current after second addition of Cr(III). Variation of the current was recorded at +250 mV vs a screen-printed Ag/AgCl reference electrode.

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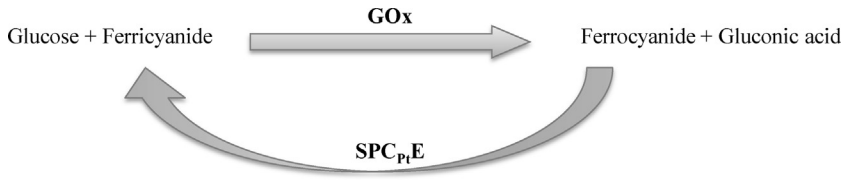

Scheme 2. Enzymatic mechanism of GOx/SPC$_{Pt}$Es biosensors.

immobilized on the electrode. For each injection, a quite fast decrease in the current (ΔI) can be observed which was proportional to the final concentration of the inhibitor. ΔI values depends on several parameters including, applied potential (E_{ap}), pyrocatechol concentration and pH of the supporting electrolyte solution. As it has been described above the recorded signal is related to the oxidation of the mediator TTF on the SPCE surface. This oxidation takes place at potentials around +250 mV [53,54]. Thus, the operational potential was set at this value and an optimization procedure was performed with the remaining variables (pH and concentration of pyrocatechol). The optimization process was carried out using an experimental design methodology. Thus, a 2.8 mM concentration of pyrocatechol and a pH of 5, were selected, using a 2^2 central composite design, as the optimum values of these parameters for the sensitive determination of Cr (III).

Under the optimum conditions, the calibration equation obtained for standard solutions containing Cr(III) concentrations from 1.8 μM up to 15.8 μM was $\Delta I = 157.8 \cdot [\mathrm{Cr(III)}] - 38.2$ ($R^2 = 0.999$). The sensitivity determined with this biosensor was 157.8 nA μM^{-1}. Several calibration curves were carried out in this linear range in order to check the performance of the biosensor. Anomalous points, which would lead to incorrect adjustments altering the precision and the capability of detection of the developed method, were eliminated by means of robust regressions performed using the Progress program [55]. Reproducibility of the method was then calculated by determining the relative standard deviation (RSD) of the slopes of the calibration curves obtained using 5 different tyrosinase/SPC$_{TTF}$Es. The RSD value obtained (5.5%) indicates a very good reproducibility in the biosensors development.

SPC$_{TTF}$Es without additional modification were used to perform control experiments. The addition of Cr(III) to the electrochemical cell, after the addition of pyrocatechol, did not alter the registered chronoamperometric current for this substrate.

The tyrosinase/SPC$_{TTF}$E biosensor developed for Cr(III) determination has also been characterized by the calculation of its capability of detection for a given probability of false positive (α) and negative (β) according to [56,57]. The average capability of detection obtained for these probability levels was 2.0 ± 0.2 μM ($n = 5$).

3.2. GOx/SPC$_{Pt}$Es biosensors for the determination of Cr(VI)

Chronoamperometric biosensors for the analysis of Cr(VI) were also constructed using GOx as the biological component. In this case, the inhibition process caused by the presence of Cr(VI) was only measurable when using SPC$_{Pt}$E. The enzyme was immobilized on a SPC$_{Pt}$E by crosslinking it using GA and BSA. The GOx/SPC$_{Pt}$Es biosensors produced a stable response corresponding to H_2O_2 oxidation [47]. This electrochemical reaction takes place at a high potential value which can lead to multiple interference effects. Thus, the use of a redox mediator is necessary in order to reduce the applied potential and thereby also possible interferences.

Different compounds were studied as possible mediators for GOx/SPC$_{Pt}$Es biosensors. From an analytical point of view, the best results were obtained when ferricyanide was added to the electrochemical measurement cell.

The chronoamperometric response of the GOx/SPC$_{Pt}$E biosensor in presence of ferricyanide can be related to the oxidation of the redox mediator according to the electrochemical process shown in Scheme 2. Fig. 2 shows a typical steady state chronoamperometric response of the GOx modified SPC$_{Pt}$E with the successive addition of Cr(VI). Analogously to the biosensor developed for the determination of Cr(III), the inhibition response can be associated with the concentration of inhibitor, Cr(VI), in this case.

The chronoamperometric determination of Cr(VI) using the developed GOx/SPC$_{Pt}$Es biosensors involves the optimization of the experimental variables that can define the sensitivity and the selectivity of these systems including the applied potential. As it can be seen in Fig. 3, the oxidation of ferricyanide takes place at a potential around +200 mV vs a screen-printed Ag/AgCl electrode. Thus, this value was selected as the optimum for the chronoamperometric analysis of Cr(VI).

Once the applied potential has been selected, the most influence experimental factors are pH value of the buffer solution, glucose concentration and ferricyanide concentration. These factors were optimized by means of a 2^3 central composite design [58], taking as response the amperometric current obtained for a concentration of 10 μM of Cr(VI). The combination of values of the factors that maximizes the response over the experimental region was,

$$\mathrm{pH} = 4;\ [\mathrm{glucose}] = 36\mathrm{mM};\ [\mathrm{ferricyanide}] = 1\mathrm{mM}$$

Under the optimum conditions, the developed biosensor showed a linear range for Cr(VI) concentration from 89 nM up to 769 nM, being the calibration equation obtained: $\Delta I = 4422 \cdot [\mathrm{Cr(VI)}] - 117$ ($R^2 = 0.999$). The sensitivity determined with this biosensor was 4422 nA μM^{-1}. Several calibration curves

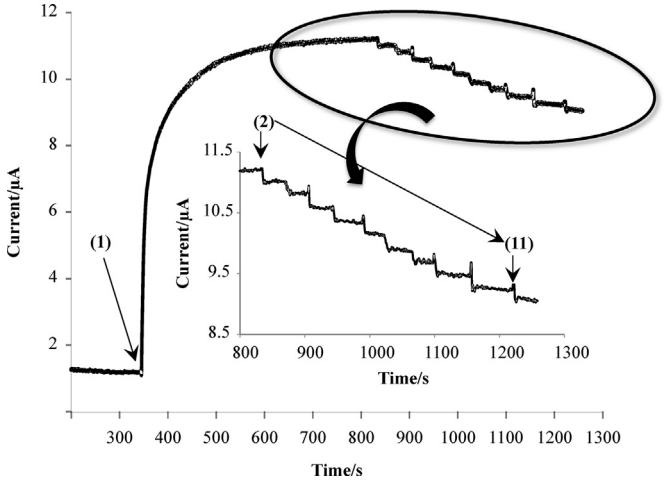

Fig. 2. Chronoamperometry experiments using a GOx/SPC$_{Pt}$E biosensor in 100 mM phosphate (pH 4) and 1 mM ferricyanide solution. (1) Addition of glucose (36 mM); (2–11) additions of 100 μL of 5 μM Cr(VI) solution. Variation of the current was recorded at +200 mV vs a screen-printed Ag/AgCl reference electrode.

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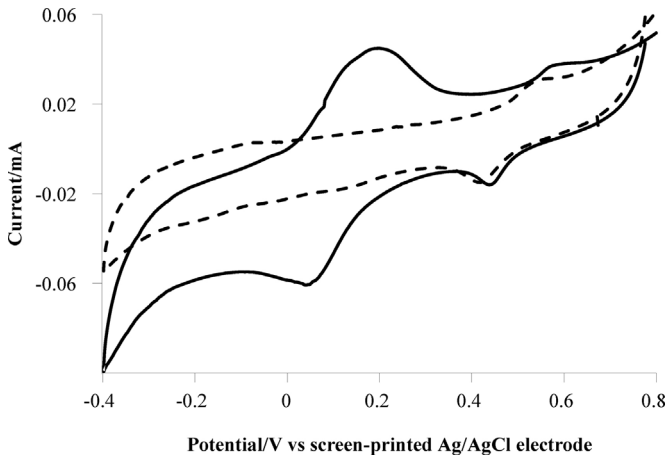

Fig. 3. Cyclic voltammograms obtained in 100 mM phosphate buffer pH 4 solution using a $SPC_{Pt}E$: blank (dot line); [ferricyanide] = 3 mM (solid line).

were carried out in this linear range in order to check the biosensor performance using robust regressions as well. Then, precision, in terms of reproducibility, and capability of detection were calculated.

The variability of the slopes of the calibration curves recorded using different electrode surfaces in the same operational conditions achieved a RSD value of 6.2% ($n = 5$). These results indicate a very good reproducibility in the biosensors development.

Non-modified $SPC_{Pt}Es$ were used to carry out control experiments. The addition of Cr(VI) to the electrochemical cell, after the addition of glucose and ferricyanide, did not modify the recorded chronoamperometric current with this electrodic system.

The GOx/$SPC_{Pt}E$ biosensor developed for Cr(VI) determination has also been characterized by the calculation of its capability of detection for a given probability of false positive (α) and negative (β) according to [56,57]. The average capability of detection obtained for these probability levels was 90.5 ± 7.6 nM ($n = 5$).

3.3. Interferences

The influence of different foreign interferents on the determination of Cr(VI) and Cr(III) was studied. Under the optimal operational conditions none of the following metallic ions analyzed – Cu(II), Ni(II), Pb(II), Cd(II), Fe(II), Fe(III), Zn(II), Sn(II), Sb(III), Sb(V), As(III) and Co(II) – produced an interfering electrochemical signal in the range of concentrations between 0.1 and 100 μM using GOx/$SPC_{Pt}Es$. As(V) and Hg(II) present an inhibition signal in a concentration level higher than 10 μM. Cr(III) not interfered in the analytical determination of Cr(VI) with this biosensor.

In the case of Cr(III) determination using $SPC_{TTF}Es$, Cu(II), Pb(II), Co(II), Zn(II), Sb(III), As(III), As(V) and Hg(II) not interfered in the range of concentrations between 0.1 μM and 100 μM. Ni(II), Fe(II), Fe(III), Sn(II) and Sb(V) present an inhibition signal in a concentration level of 1 μM and Cd(II) present an inhibition signal in a concentration level of 10 μM. Cr(VI) not interfered in the analytical determination of Cr(III) with this biosensor.

3.4. Analytical application

According to the above described results, the simultaneous analysis of Cr(III) and Cr(VI) in one step has been performed by using two different electrodes (tyrosinase/$SPC_{TTF}E$ and GOx/$SPC_{Pt}E$) connected in array mode.

First, a deep study of the suitable experimental conditions for the simultaneous determination of both chromium species was performed. In this way, it was found that the presence of chloride significantly affects the analytical response for Cr(VI) using GOx based biosensors. However, this compound is necessary for the determination of Cr(III) using tyrosinase biosensors. Several experiments were then carried out in order to determine which concentration of chloride allowed the simultaneous determination of both species. In this way, a 50 mM concentration of KCl was required to observe both inhibition responses with enough analytical quality. In the same way, the influence of pH was analyzed founding that a value of 4 for this parameter was adequate for the simultaneous determination of Cr(III) and Cr(VI). In fact, for Cr(VI), low inhibition response was obtained at the optimum pH value obtained for Cr(III) (pH 5).

The viability of the developed array biosensor was checked in the determination and speciation of the content of chromium in samples of tap water, using the method of standard addition. Tap water was spiked with Cr(III) and Cr(VI) to provide a 1.8 mM solution. Then, 100 μL of the spiked water sample were placed into the electrochemical cell, following by successive additions of substrates and chromium solution, according to the procedure described in Section 2.6. The concentration detected using the array biosensor was 1.9 ± 0.1 mM and 1.8 ± 0.1 mM for Cr(III) and Cr(VI), respectively. On the basis of these results, it can be concluded that the developed method can be considered appropriate in the speciation of chromium in tap water samples.

The analysis of a sample obtained as residue of a tanning industry has been also made. The concentrations of Cr(III) and Cr(VI) found using the developed bipotentiostatic chronoamperometric biosensor were 0.45 ± 0.02 mM ($n = 3$, $\alpha = 0.05$) and 1.3 ± 0.06 μM ($n = 3$, $\alpha = 0.05$), respectively. These results were checked using an electrochemical reference method based on adsorptive stripping differential pulse voltammetry [38]. The concentrations of Cr(III) and Cr(VI) found, 0.47 ± 0.06 mM ($n = 3$, $\alpha = 0.05$) and 1.2 ± 0.1 μM ($n = 3$, $\alpha = 0.05$), respectively, are in good agreement with the ones obtained with the new developed.

4. Conclusions

The viability of the determination of Cr(III) in presence of Cr(VI) using tyrosinase/$SPC_{TTF}E$ biosensors has been successfully demonstrated. The selective developed biosensor shows high reproducibility and sensitivity levels. In the same way, the sensitive determination of Cr(VI) in presence of Cr(III) has been performed using GOx/$SPC_{Pt}E$. Both developed sensors show a high level of selectivity towards foreign metals due to the use of redox mediators. This fact represents an important advantage from previously described chronoamperometric biosensors, which used higher operational potentials [46,47]. In the case of tyrosinase/$SPC_{TTF}E$ biosensors for Cr(III), the screen-printed TTF mediator reagent allows the simpler manufacture as well as decreasing the operational potential. The described biosensors present advantages of electrochemical techniques, such as simplicity and low cost, along with those added by the employ of disposable SPEs.

Finally, the novel described array biosensors resulted to be appropriate to the simultaneous determination of Cr(III) and Cr(VI) in the same sample including complex matrices such as tap water and waste water from a tannery. The developed method involves a very simple procedure for carrying out the speciation of chromium.

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