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## Electrocatalytic oxidation of thiocholine at chemically modified cobalt hexacyanoferrate screen-printed electrodes

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## ABSTRACT

In this work, cobalt hexacyanoferrate (CoHCF) modified screen-printed electrodes were developed and characterised electrochemically. The effect of different cations was investigated showing a more reversible system with a well-defined peak around +0.35 V vs. Ag/AgCl when Na<sup>+</sup> was used as cation in the supporting electrolyte. The transfer coefficient ( $\alpha$ ) and the apparent charge transfer rate constant ( $k_s$ ) for electron transfer between the electrode and the CoHCF layer was calculated. The electrocatalytic behaviour of CoHCF towards oxidation of enzymatically generated thiocholine was thoroughly investigated. Cyclic voltammetry experiments performed in thiocholine solution showed the classical shape of a mediated redox system. The diffusion constant of thiocholine was estimated using chronoamperometry. Also, the catalytic rate constant  $k$  of thiocholine at CoHCF-SPE was determined. The current due to thiocholine mediated oxidation was then evaluated in batch amperometric mode at +0.5 V vs. Ag/AgCl obtaining a linear range ( $5 \times 10^{-7}$ – $1 \times 10^{-5}$  mol L<sup>-1</sup>) with a low detection limit and a high sensitivity equal to  $5 \times 10^{-7}$  mol L<sup>-1</sup> and 435 mA mol<sup>-1</sup> L cm<sup>-2</sup>, respectively.

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### 1. Introduction

Thiocholine is the product of thiocholine ester (TChE) hydrolysis catalysed by cholinesterase enzymes ChEs (acetylcholinesterase, AChE, or butyrylcholinesterase, BChE). The detection of thiocholine is important as a means to evaluate the enzymatic activity of ChEs, enzymes that can be used as biocomponent of biosensor for detection of organophosphorous and carbammic pesticides [1–3], nerve agents [4,5] or, as demonstrated recently by our group, for aflatoxin B detection [6]. For future application of biosensors for environmental and food analysis, sensitive, fast and cost-effective sensors for thiocholine detection are required.

In order to measure thiocholine, spectrophotometric detection is often carried out using Ellman's method [7–9] which is based on the use of the thiol–disulfide exchange reaction between thiocholine (thiol) and 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB, disulfide). The sulfide produced in the reaction is the 5-thio-2-nitrobenzoic acid (TNB), which has a maximum absorption at

412 nm ( $\epsilon = 13.600$  mol<sup>-1</sup> L cm<sup>-1</sup>). Relative to spectrophotometric determination, electroanalysis presents several advantages such as high sensitivity, simplicity, real time detection and the potential to miniaturize the analytical system. Moreover, the measurements can often be carried out by no skilled personnel. However, the direct oxidation of thiocholine at conventional electrodes, such as those made of glassy carbon, carbon paste or platinum, is slow and requires high overpotentials with the added problem of electrode surface fouling. Different mediators have been investigated as to their ability to overcome these drawbacks and to enable an amperometric detection of thiocholine at low applied potentials without electrode passivation. Among the mediators used for thiocholine detection, metal phthalocyanine such as ferro phthalocyanine and cobalt phthalocyanine were investigated demonstrating an electrocatalytic response for cholinesterase–thiocholine ester system [10]. However, cobalt phthalocyanine respect to ferro phthalocyanine remains one of the most frequently used electrocatalysts for this purpose due to its low detection limit (equal to  $5 \times 10^{-7}$  mol L<sup>-1</sup>) and the ease of production of the modified sensor [11,12].

Thiocholine has also been determined using TCNQ (7,7',8,8'-tetracyanoquinodimethane) as electrochemical mediator. The measurement is carried out by the differential pulse voltammetry technique reaching a detection limit equal to  $2.5 \times 10^{-6}$  mol L<sup>-1</sup>.

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This sensor was subsequently used as substrate for a ChE biosensor with applications in the food and environmental fields [13–15].

Recently, ferric hexacyanoferrate (Prussian Blue) has also been shown to possess electrocatalytic properties towards thiocholine detection [16]. High amperometric signals, with a corresponding low detection limit ( $5 \times 10^{-6} \text{ mol L}^{-1}$ ), were obtained for thiocholine at low applied potential (+0.2 V vs. Ag/AgCl). The ease of surface modification together with the high stability of Prussian Blue allowed the use of this sensor as device for the development of a ChE biosensor for pesticide detection in river water and in grape juice [17,18].

Some hexacyanoferrates were adopted as electrochemical mediators for thiol compounds such as copper, nickel and cobalt hexacyanoferrate. Glassy carbon modified with copper hexacyanoferrate was developed and characterised by Chen and Chan [19] demonstrating the ability to electrocatalyse the oxidation of cysteine and glutathione at applied potential around +0.8 V vs. Ag/AgCl. The paraffin impregnate graphite electrode was mechanically modified with copper hexacyanoferrate or nickel hexacyanoferrate by Ravi Shankaran and Sriman Narayanan [20,21] showing the possibility to detect some thiols such as cysteine or glutathione with good reproducibility. The cobalt hexacyanoferrate and nickel hexacyanoferrate have shown a formal potential around +0.4 V vs. Ag/AgCl lower than applied potential using copper hexacyanoferrate [19–21], in addition lower detection limits were obtained using cobalt hexacyanoferrate for glutathione, cysteine and N-acetylcysteine equal to  $2.5 \times 10^{-6} \text{ mol L}^{-1}$ ,  $1 \times 10^{-6} \text{ mol L}^{-1}$  and  $1.5 \times 10^{-6} \text{ mol L}^{-1}$ , respectively [22].

However, in the literature only ferric hexacyanoferrate was reported to have electrocatalytic properties towards thiocholine oxidation. In order to evaluate, other hexacyanoferrates as electrochemical mediators for thiocholine detection, we have chosen to investigate cobalt hexacyanoferrate (CoHCF), because (1) cobalt phthalocyanine have shown a major application than ferro phthalocyanine as electrochemical mediator for thiocholine detection as reported in the literature [10–12], thus we have investigated CoHCF in order to evaluate if also for hexacyanoferrate compounds the presence of cobalt can improve the analytical performance of the modified electrode; (2) in addition it can be considered an attractive compound for electrochemical measurements due to its excellent reversible redox centers, in fact CoHCF modified electrodes have been widely used to electrocatalyze the oxidation of a variety of important molecules such as ascorbic acid [23], dopamine [24], thiosulfate, nitrite, hydrazine, hydroxylamine, p-chlorophenol [25,26] and morphine [27]. To our knowledge this is the first time that the electrochemical behaviour of CoHCF toward thiocholine has been investigated. Screen-printed electrodes were chemically modified with CoHCF using a simple method and the electrochemical characterisation of these CoHCF–SPEs was carried out. Using cyclic voltammetry and chronoamperometry techniques, the electrocatalytic activity of CoHCF toward thiocholine was demonstrated. The analytical performance was also investigated using the amperometric technique.

## 2. Experimental

### 2.1. Apparatus

Amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to a X–t recorder (L250E, Linseis, Selb, Germany).

Cyclic voltammetry (CV) was performed using an Autolab electrochemical system (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands).

### 2.2. Electrodes

Screen-printed electrodes (SPEs) were home produced with a 245 DEK (Weymouth, England) screen printing machine and using different inks obtained from Acheson (Milan, Italy). Graphite-based ink (Electrodag 421), silver ink (Electrodag 477 SS RFU), and insulating ink (Electrodag 6018 SS) were used; the substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The electrodes were produced in foils of 48. The diameter of the working electrode was 0.3 cm resulting in an apparent geometric area of  $0.07 \text{ cm}^2$ . The silver ink was used to print the reference electrode. Before thiol measurements, the reference electrode was chlorurated. To do this, a potential of +0.6 V was applied between the silver ink and an external Ag/AgCl electrode for 20 s in a phosphate buffer solution in the presence of  $0.1 \text{ mol L}^{-1}$  KCl [28].

### 2.3. Reagents

All chemicals from commercial sources were of analytical grade.

Acetylcholinesterase (AChE) from electric eel (EC 3.1.1.7, 244 units/mg), acetylthiocholine chloride and DTNB were obtained from Sigma (St. Louis, MO). The solution of DTNB ( $1 \text{ mmol L}^{-1}$ ) used in the experiments was prepared in phosphate buffer  $10 \text{ mmol L}^{-1}$ , pH 7.0. For pH measurements, a Britton–Robinson universal (4–12 pH) buffer ( $0.05 \text{ mol L}^{-1}$  of phosphate, acetate and borate sodium salts) was used; pH was adjusted using KOH and HCl.

### 2.4. Thiocholine production

Since, thiocholine is not commercially available, it was produced enzymatically using AChE as enzyme and acetylthiocholine as substrate. For this purpose, 1 mL of acetylthiocholine solution,  $1 \text{ mol L}^{-1}$ , was prepared in phosphate buffer  $0.1 \text{ mol L}^{-1}$  (pH = 8) and 100 units of AChE were added to this solution. After 1 h, the concentration of thiocholine produced by AChE was estimated spectrophotometrically by Ellman's method. For this purpose,  $900 \mu\text{L}$  of phosphate buffer solution ( $0.1 \text{ mol L}^{-1}$ , pH = 8),  $100 \mu\text{L}$  of  $0.1 \text{ mol L}^{-1}$  DTNB and  $5 \mu\text{L}$  thiocholine solution (diluted 1:100 in water) were put in a cuvette. Absorbance was measured at different times and the real concentration was evaluated using the Lambert–Beer law with the known molar extinction coefficient of TNB ( $\epsilon = 13,600 \text{ mol}^{-1} \text{ L cm}^{-1}$ ) [7]. After 1 h, the hydrolysis reaction of acetylthiocholine is completed and a 1 mL solution of  $1 \text{ mol L}^{-1}$  thiocholine is obtained. The solution is stable at  $4^\circ\text{C}$  for one day. To use the same solution the next day, the exact concentration of thiocholine was again evaluated using the spectrophotometric Ellman's test, before amperometric measurement.

### 2.5. Preparation of CoHCF modified screen-printed electrodes

CoHCF modification of SPEs was accomplished by placing two drops ( $10 \mu\text{L}$  total volume) of “precursor solutions” onto the working electrode area. Five microliters of  $0.1 \text{ mol L}^{-1}$  sodium ferrocyanide ( $\text{Na}_4\text{Fe}(\text{CN})_6$ ) in  $10 \text{ mmol L}^{-1}$  HCl and  $5 \mu\text{L}$  of  $0.1 \text{ mol L}^{-1}$  cobalt chloride ( $\text{CoCl}_2$ ) in  $10 \text{ mmol L}^{-1}$  HCl were carefully applied exclusively on the working electrode area. After 1 h, the SPEs were rinsed with a few millilitres of  $10 \text{ mmol L}^{-1}$  HCl. The probes were then left 90 min in the oven at  $100^\circ\text{C}$  to obtain a more stable layer of CoHCF. The CoHCF modified electrodes were stored under vacuum at room temperature in the dark.

### 2.6. Amperometric thiocholine measurements

Measurements of thiocholine were performed using amperometric batch analysis in a stirred phosphate buffer solution

0.05 mol L<sup>-1</sup> + NaCl 0.1 mol L<sup>-1</sup>, pH 7.0 (10 mL) with an applied potential of +0.5 V vs. Ag/AgCl. When a stable baseline current was reached, the analyte was added and the response was recorded.

### 3. Results and discussion

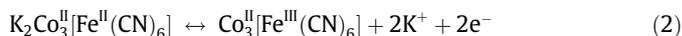
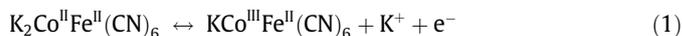
#### 3.1. Electrochemical behaviour of the modified electrode

The preparation of the CoHCF modified SPEs (CoHCF-SPEs) was carried out using the chemical deposition because using Biodot<sup>®</sup> dispenser machine [29] it is possible to automatise the production of modified SPE. In this way, with both SPEs and chemical deposition it is possible to have a fast and economic mass production of thiocholine sensor. The chemical deposition of CoHCF involves two steps, firstly the deposition of 5 μL of sodium ferrocyanide and then the deposition of 5 μL cobalt chloride on the working electrode of SPEs. The optimisation of deposition procedure was carried out studying the effect of different concentrations of each precursor (0.05 mol L<sup>-1</sup>, 0.1 mol L<sup>-1</sup>, 0.2 mol L<sup>-1</sup>, 0.5 mol L<sup>-1</sup> and 1 mol L<sup>-1</sup>) and the time of deposition (from 10 min up to 120 min). The best condition to obtain good cyclic voltammetry behaviour (Fig. 1) was: sodium ferrocyanide 0.1 mol L<sup>-1</sup>, cobalt chloride 0.1 mol L<sup>-1</sup> and 60 min as deposition time. Fig. 1 shows the cyclic voltammetry before and after the chemical modification of SPEs with CoHCF. The voltammogram exhibits a pair of peaks (peaks potential:  $E_{pa} = 0.353$  V and  $E_{pc} = 0.324$  V) with formal potential  $(E_{pa} + E_{pc})/2$  equal to 0.339 V and the peak to peak separation ( $\Delta E_p$ ) at low scan rate (40 mV/s) is about 30 mV.

The difference of the experimental  $\Delta E_p$  from the ideal surface redox process ( $\Delta E_p = 0$ ) is due to limitations associated with charge propagation in the film, the chemical interaction between the ions and the modified film, and the polarizability of the cation influencing its penetration in or out of the CoHCF lattice or non equivalent sites in the film.

In order to maintain the electroneutrality of the film during the redox reaction, the ions will enter or exit from the film and thus the ions play an important role during this process. The effect of three different alkali metal cations was investigated using CV. Fig. 2 shows the cyclic voltammograms using the CoHCF-SPE in a 0.1 mol L<sup>-1</sup> solution of NaCl (a), KCl (b) and LiCl (c). Using K<sup>+</sup> as alkali metal cation a pair of well-defined peaks with a formal potential equal to 0.475 V and another pair of peaks near 0.6 V were observed. In this latter case, the cathodic peak is better defined

than the anodic peak. This behaviour is similar to the results obtained from Tao et al. [30] using CoHCF films immobilised on Au-colloid modified gold electrode with KNO<sub>3</sub> as supporting electrolyte. In the case of the Li as the alkali metal ion, two different redox couples were also obtained having the formal potentials of 0.274 and 0.488 V, respectively. The first peak was assigned to the Co<sup>II</sup>/Co<sup>III</sup> which is also supported by similar reports of cobalt phthalocyanine and the second to Fe<sup>II</sup>(CN)<sub>6</sub>/Fe<sup>III</sup>(CN)<sub>6</sub> transition which analogous to the those reported for Prussian Blue. For example in presence of K<sup>+</sup> the redox system can be described by the following equations:



as demonstrated by Lezna et al. [31] using in situ IR spectroelectrochemistry spectroscopy.

On the contrary, in the case of Na<sup>+</sup> as alkali metal ion, a single well-defined redox couple was observed with the formal potential of 0.367 V. Chen [32], in fact, has demonstrated that two different types of CoHCF have been synthesised: one type with two redox couples (when the CoHCF was synthesised with KNO<sub>3</sub> or LiNO<sub>3</sub> as supporting electrolyte) and another with a single redox couple (if the CoHCF was synthesised using NaNO<sub>3</sub> as supporting electrolyte). Recently, a study of different types of CoHCF deposition on carbon film electrode was carried out by Pauliukaite et al. [33]. In this study, the chemically deposition has exhibited one redox couple and this couple can be probably attribute to the summation of the both processes Co<sup>II</sup> ↔ Co<sup>III</sup> and Fe<sup>II</sup> ↔ Fe<sup>III</sup>, while when the film was deposited by potential cycling, two redox of couples are present in CV. The first of these at less positive potential and the second (a small peak) at higher than couple of peaks obtained using the electrode chemically modified. Thus, this means that the structure of the film changes according to the deposition method.

In our case as shown in Fig. 1 a second reversible process around 0.5 V vs. Ag/AgCl can be observed, probably due to Fe<sup>II</sup> ↔ Fe<sup>III</sup> couple that can be probably distinguished from Co<sup>II</sup> ↔ Co<sup>III</sup> couple in NaCl 0.1 mol L<sup>-1</sup> solution.

In addition, we have observed that in the redox reaction of CoHCF just one electron is involved in fact the peak half width ( $W_{h1/2}$ ) is ca. 90 mV, value in agreement with mono-electron transfer reaction [34], this means that redox couple at 0.339 V can be probably attribute to the Co<sup>II</sup> ↔ Co<sup>III</sup> couple.

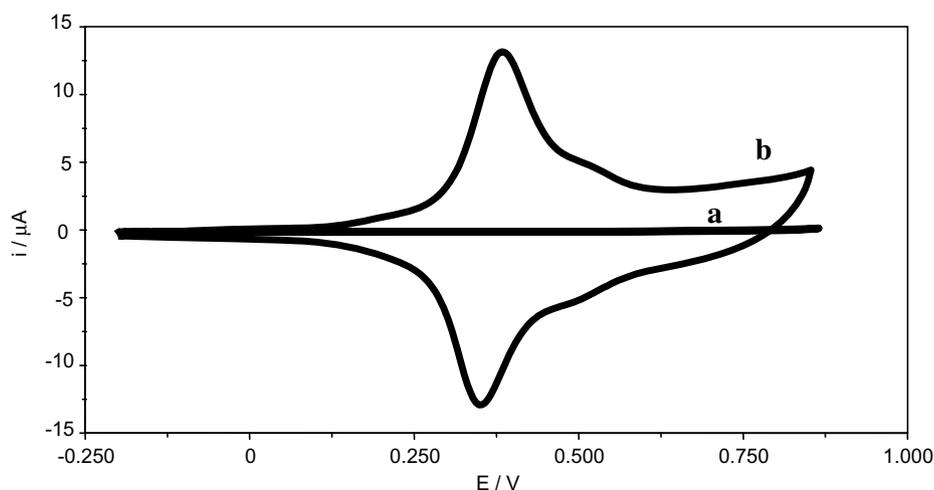


Fig. 1. Cyclic voltammograms revealing the chemical deposition of CoHCF on SPEs. The cyclic voltammogram obtained with unmodified electrodes is also shown. Scan rate 50 mV/s: (a) unmodified electrode in NaCl 0.1 mol L<sup>-1</sup> and (b) CoHCF modified electrode in NaCl 0.1 mol L<sup>-1</sup>.

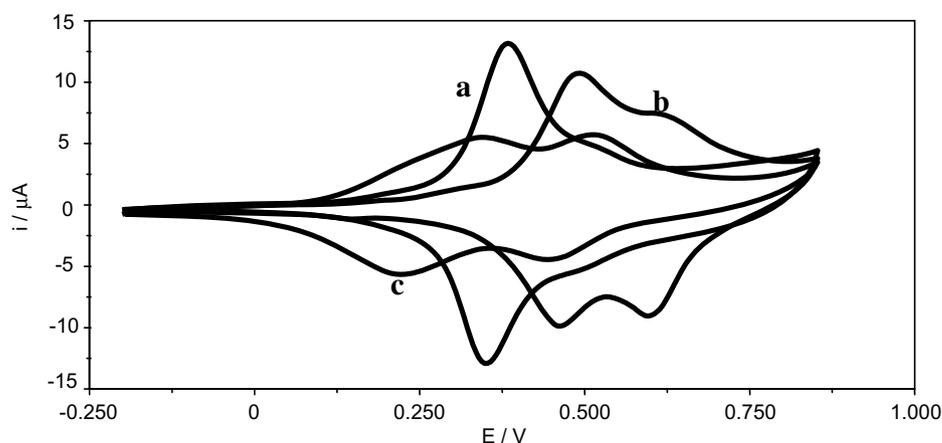


Fig. 2. Cyclic voltammograms of a CoHCF-SPE in a 0.1 mol L<sup>-1</sup> (a) NaCl, (b) KCl and (c) LiCl. Scan rate 50 mV/s.

On the basis of CV using our conditions, the CoHCF-SPE used with NaCl showed a better system reversibility, with a lower  $\Delta E_p$ , and higher peak currents than that obtained using KCl or LiCl as supporting electrolyte. In addition, the major stability was observed using NaCl as supporting electrolyte probably due to the fact that our optimised deposition involves the use of sodium ferrocyanide. In fact, as reported in the literature [25] the CoHCF film prepared in presence of Na<sup>+</sup>, shows a different working stability. The CoHCF has characterised by good stability if the electrolyte is a solution with Na<sup>+</sup> as cation, instead the film easily redissolved in electrolyte containing K<sup>+</sup> or Li<sup>+</sup>. For this reason, the NaCl was adopted as supporting electrolyte for the rest of the work.

The electrochemical behaviour of the SPE modified with CoHCF was then investigated using the cyclic voltammetry technique. The CV of CoHCF-SPEs was carried out at different scan rates between -0.2 and 0.9 V vs. Ag/AgCl (Fig. 3). The current of the anodic and cathodic peaks increases linearly with increasing scan rate up to 200 mV/s, demonstrating that there is a surface controlled electrochemical reaction (Fig. 3b). In fact, for thin films and low scan rates (if finite diffusion occurs within the film), thin-layer behaviour predominates, and the peak current is proportional to  $\nu$  according to the following Eq. (3) [34]:

$$I_p = (n^2 F^2 / 4RT) \nu A \Gamma \quad (3)$$

where  $\nu$  is the scan rate,  $n$  is the number of the electrons involved in the redox reaction,  $F$  is the Faraday constant,  $A$  is the surface of the working electrode assuming that the surface electrode corresponds to the geometrical area of the SPE and  $\Gamma$  is surface coverage. In contrast, for high scan rates from 250 mV/s up to 1000 mV/s, the peak currents are proportional to  $\nu^{1/2}$  due to the fact that the electrochemical process is controlled by diffusion of the counter ion (Na<sup>+</sup>) (Fig. 3c).

### 3.2. Charge transfer rate in the film

CoHCF-SPE surface coverage was calculated from the CVs at three different scan rates (2, 20 and 40 mV/s) using Eq. (3) and from the following equation  $\Gamma = Q/nFA$  [36] where  $Q$  is the area under the anodic peak. A value of  $6.0 \times 10^{-9}$  mol cm<sup>-2</sup> was obtained and is comparable to the surface coverage found by Cai et al. [35] and Pournaghi-Azar and Sabzi [36].

For the CoHCF-SPE with this surface coverage, the peak to peak separation potential observed for scan rates up to 100 mV/s is less than 50 mV while at scan rates higher than 100 mV/s (Fig. 3a) a wider separation appears (177 mV for 1 V/s), indicating the limitations arising from charge transfer kinetics. The transfer coefficient

( $\alpha$ ) and the apparent charge transfer rate constant ( $k_s$ ) for electron transfer between the electrode and the CoHCF layer using Na<sup>+</sup> as supporting electrolyte cation was calculated using Laviron theory for surface-confined electroactive species [37]

$$E_{pa} = E^\circ + A \ln[(1 - \alpha)/m] \quad (4)$$

$$E_{pc} = E^\circ + B \ln[\alpha/m] \quad (5)$$

$$\text{For } \Delta E_p > 200/n \text{ (mV)}$$

$$\log k_s = \alpha \log(1 - \alpha) + (1 - \alpha) \log \alpha - \log(RT/nF\nu) - \alpha(1 - \alpha)nF\Delta E_p/2.3RT \quad (6)$$

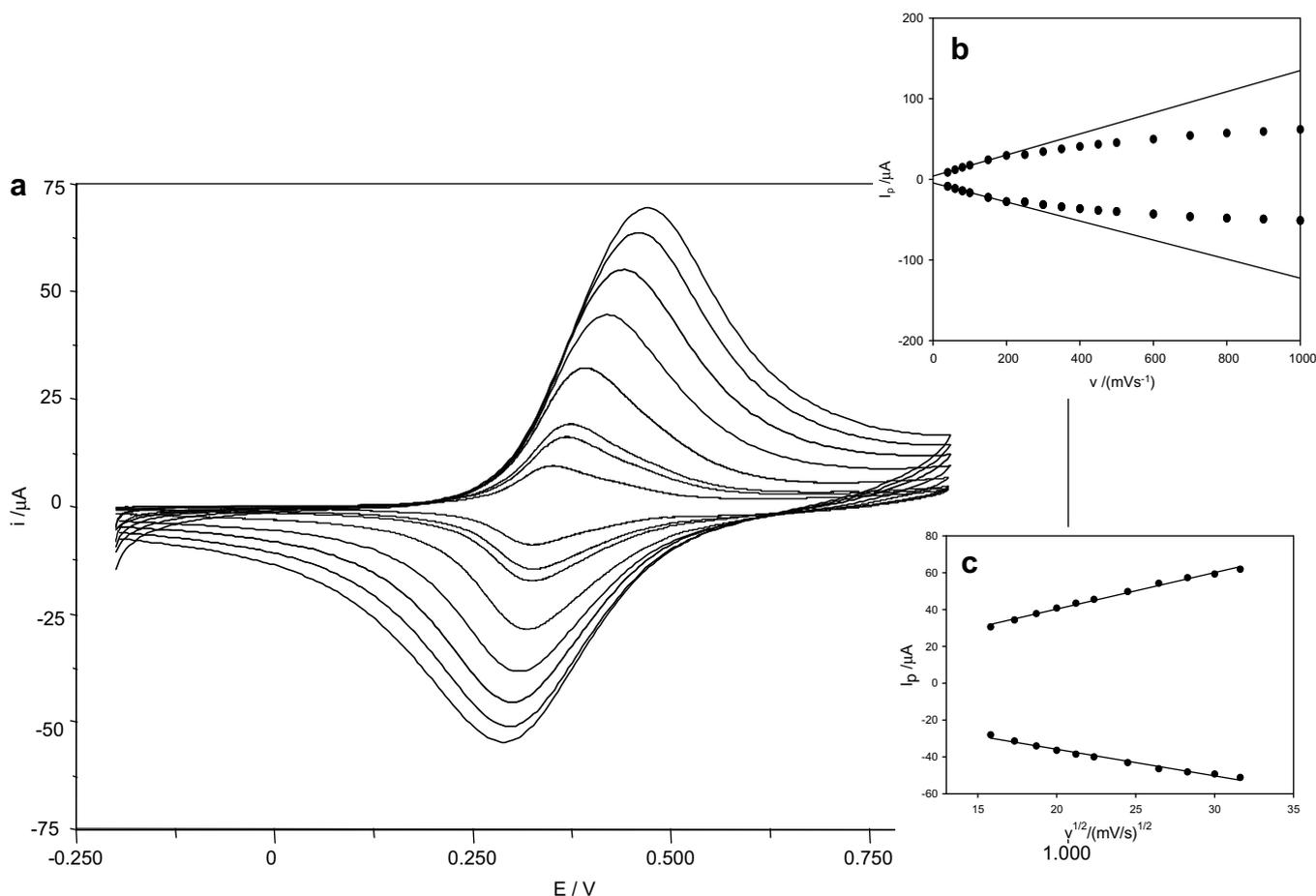
where  $A = RT/(1 - \alpha)nF$ ,  $B = RT/\alpha nF$  and  $m = (RT/F)(k_s/n\nu)$ .

By plotting  $E_p - E_0$  as a function of  $\log \nu$  (Fig. 4), two straight lines with slopes equal to 2.3A and 2.3B for the cathodic and the anodic peaks, respectively, are obtained. We have found that for a high scan rate from 2 to 5 V/s, and using the Na<sup>+</sup> as cation in the supporting electrolyte, the  $\log \nu$  is proportional to  $E_p - E_0$  in accord with Laviron theory. The  $\alpha$  and the  $k_s$  were calculated as reported above and found to be equal to 0.5 and 1.53 s<sup>-1</sup>, respectively. These values are in agreement with the values obtained by Pournaghi-Azar and Sabzi [36] who have studied these parameters using a CoHCF film electrochemically prepared on aluminium electrodes.

### 3.3. Electrochemical characterisation of thiocholine at a CoHCF-SPE

The behaviour of CoHCF toward thiocholine oxidation was investigated using a CV technique. Fig. 5 shows the CVs using the unmodified and modified CoHCF-SPE in the absence and in the presence of thiocholine. In the case of CV using unmodified electrode a negligible difference was observed until +0.6 V vs. Ag/AgCl, in fact using bare electrode the thiocholine oxidation starts at around +0.7 V vs. Ag/AgCl (Fig. 5). On the contrary, in presence of thiocholine (RSH) in phosphate buffer using CoHCF-SPE the current due to the CoHCF oxidation increases with respect to the scan obtained in the absence of thiocholine while in the reverse scan, in presence of thiocholine, the current of the cathodic peak due to the CoHCF reduction decreases as expected for mediated oxidative reactions. These results seem to demonstrate that the CoHCF has electrocatalytic activity towards thiocholine oxidation. The generic reaction can be described by the following equations:





**Fig. 3.** Cyclic voltammograms using the CoHCF–SPE in Britton–Robinson buffer + NaCl 0.1 mol L<sup>-1</sup> pH = 4 at different scan rates: 40, 80, 100, 200, 400, 600, 800 and 1000 mV/s (Fig. 3a). Inset plot (Fig. 3b) of anodic and cathodic peak currents vs. potential scan rate (40, 60, 80, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900 and 1000 mV/s). Inset plot of anodic and cathodic peak currents vs. square root of the potential scan rate (Fig. 3c).

According to the above equations, the addition of the thiocholine causes an increase in concentration of the CoHCF<sub>red</sub> at the surface of working electrode, resulting in an increase of the anodic peak current. On the contrary, the cathodic peak current is proportional to CoHCF<sub>ox</sub> at the surface of working electrode, a quantity that decreases after the addition of RSH. Moreover, increasing the thiocholine concentration led to a further increase of the anodic peak current and a decrease of the cathodic peak, confirming the property of CoHCF as electrocatalyst for thiocholine oxidation.

The catalytic rate constant  $k$  was then calculated using the chronoamperometric technique and the following equation (Eq. (9)) [38]:

$$I_{\text{cat}}/I_L = \pi^{1/2}(kCt)^{1/2} \quad (9)$$

where  $I_{\text{cat}}$  and  $I_L$  are the currents in the presence and in the absence of thiocholine, respectively;  $k$  is the heterogenous electron transfer rate constant,  $C$  is the concentration of thiocholine and  $t$  is the time at which the current values are taken. Plotting the ratio  $I_{\text{cat}}/I_L$  vs.  $t^{1/2}$ , resulted in a straight line (Fig. 6), and from its slope the rate constant was calculated. Using a concentration of thiocholine equal to 3 mmol L<sup>-1</sup>, a value of 89 mol<sup>-1</sup> L s<sup>-1</sup> was obtained as catalytic rate constant. The diffusion coefficient ( $D$ ) for thiocholine has been calculated using the chronoamperometric technique and the Cottrell equation (Eq. (10)) [39]

$$I = nFAD^{1/2}C/\pi^{1/2}t^{1/2} \quad (10)$$

In Fig. 7a the chronoamperograms are shown and from these data the plot of  $I$  vs.  $t^{-1/2}$  can be constructed for different concentrations of thiocholine (Fig. 7b). The slopes of the resulting straight lines were then plotted vs. thiocholine concentration (Fig. 7c) and from the slope of this latter line, the diffusion coefficient for thiocholine was calculated as equal to  $6.0 \times 10^{-7}$  cm<sup>2</sup> s<sup>-1</sup>. To our best knowledge, this is the first time that the diffusion coefficient and the catalytic rate constant  $k$  for the thiocholine reaction with CoHCF has been calculated.

### 3.4. Study of pH-dependence

In order to choose the best conditions for thiocholine detection, the effect of pH on the electroactivity of the mediator was investigated. In the literature it is reported that hexacyanoferrates show the best electrochemical performance at acid pH [40]. We have investigated a large range of pH from 4 to 10 using a Britton–Robinson buffer. Similar electrochemical behaviours were obtained in the range from 4 to 7. A different behaviour was instead observed at pH = 9 and 10; at basic pH (i.e. pH = 9) the film is not stable and after 5 voltammetric cycles, a decrease of peak current of around 50% was observed, by contrast, at neutral or acid pHs, the CV current showed higher stability in fact after 50 CVs only a decrease of 6% was observed (data not shown). This effect is probably due to the interaction between ferric ions and hydroxyl groups which form a soluble Fe(OH)<sub>3</sub> which in turn degrades the film [40]. Thus,

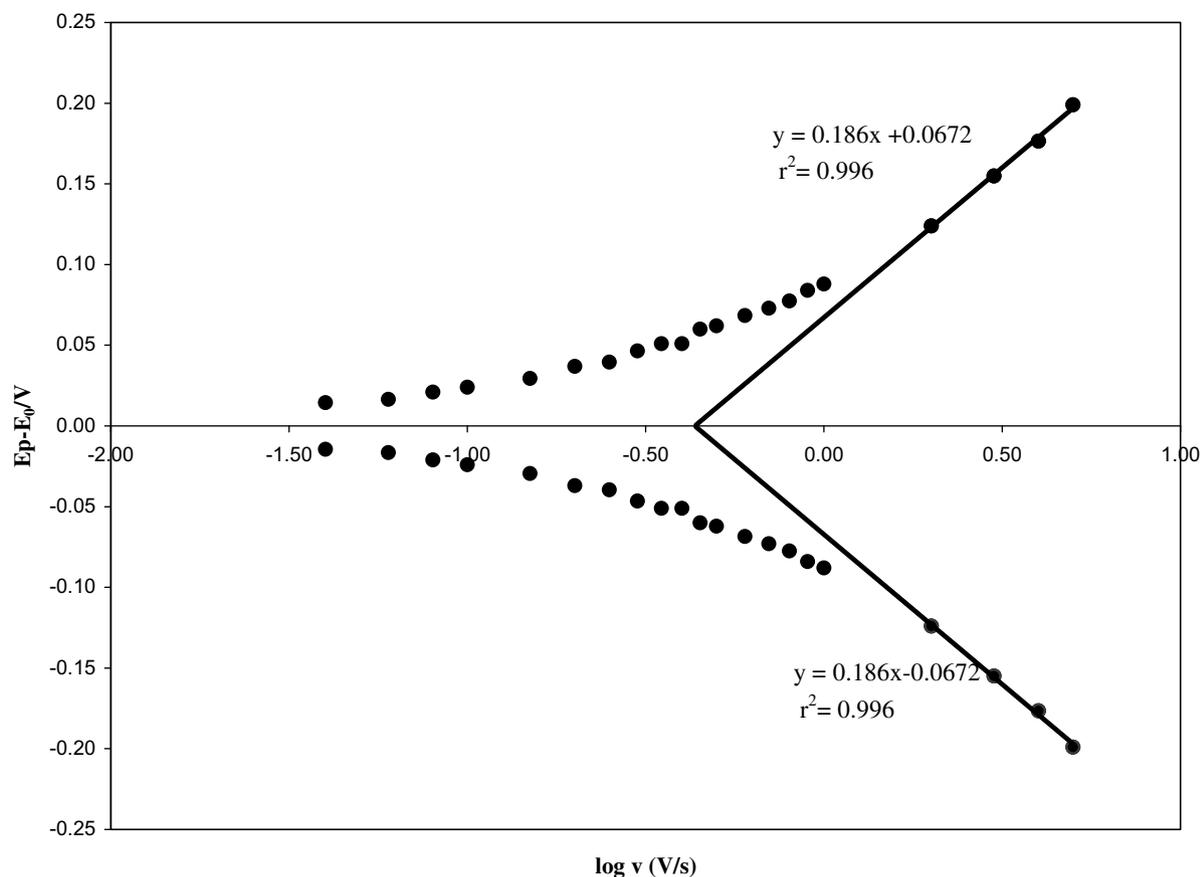


Fig. 4. Plot of  $E_p - E_0$  vs.  $\log v$  for cyclic voltammograms recorded with CoHCF-SPE in Britton-Robinson buffer + NaCl 0.1 mol L<sup>-1</sup>, pH = 4.

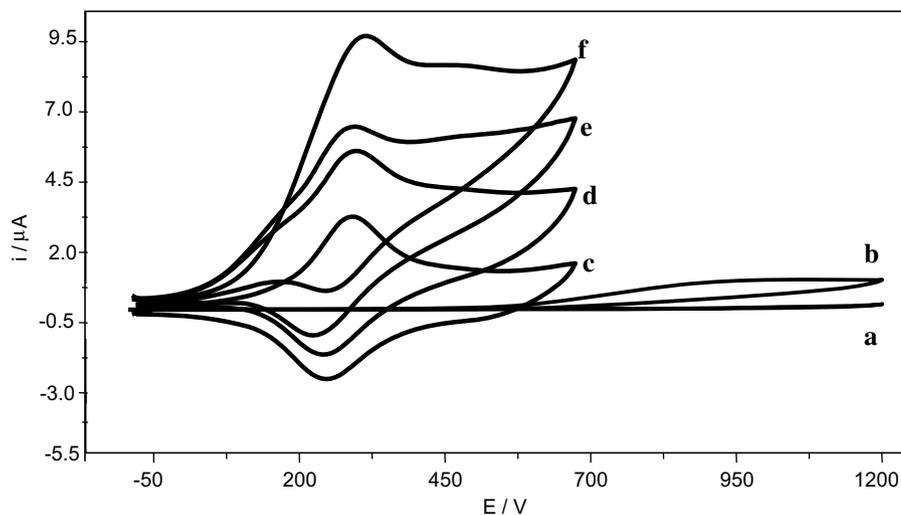


Fig. 5. Cyclic voltammograms revealing the catalytic oxidation of thiocholine at a CoHCF-SPE. Cyclic voltammograms obtained with unmodified electrodes are also shown. Phosphate buffer 0.05 mol L<sup>-1</sup> + NaCl 0.1 mol L<sup>-1</sup>, pH 7.0. Unmodified electrode in phosphate buffer (a) and in thiocholine 5 mmol L<sup>-1</sup> (b). CoHCF-SPE in phosphate buffer (c), and in presence of 1 (d), 3 (e) and 5 mmol L<sup>-1</sup> (f) of thiocholine. Scan rate 100 mV/s.

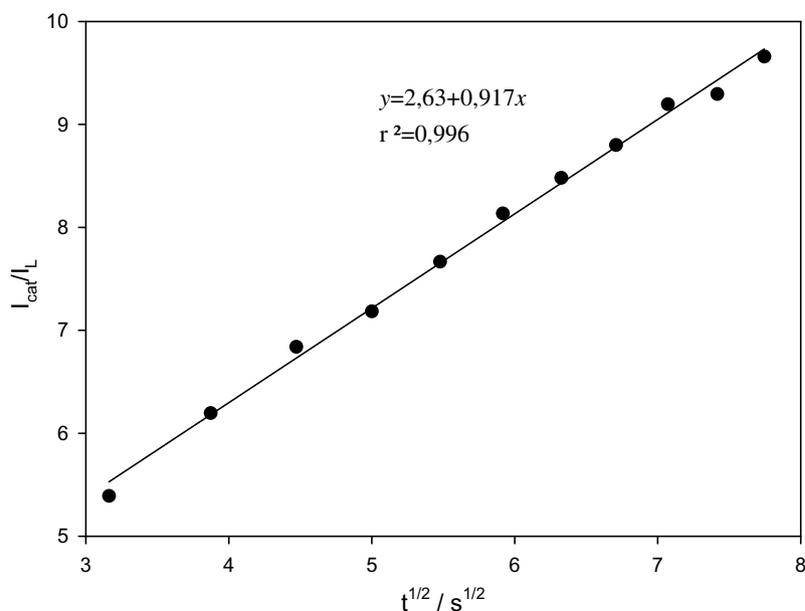
the stability study shows that the CoHCF-SPEs can be used at acid or neutral pHs with good working stability.

Because the sensor is to be used for thiocholine detection, the pH effect on thiocholine oxidation was also evaluated.

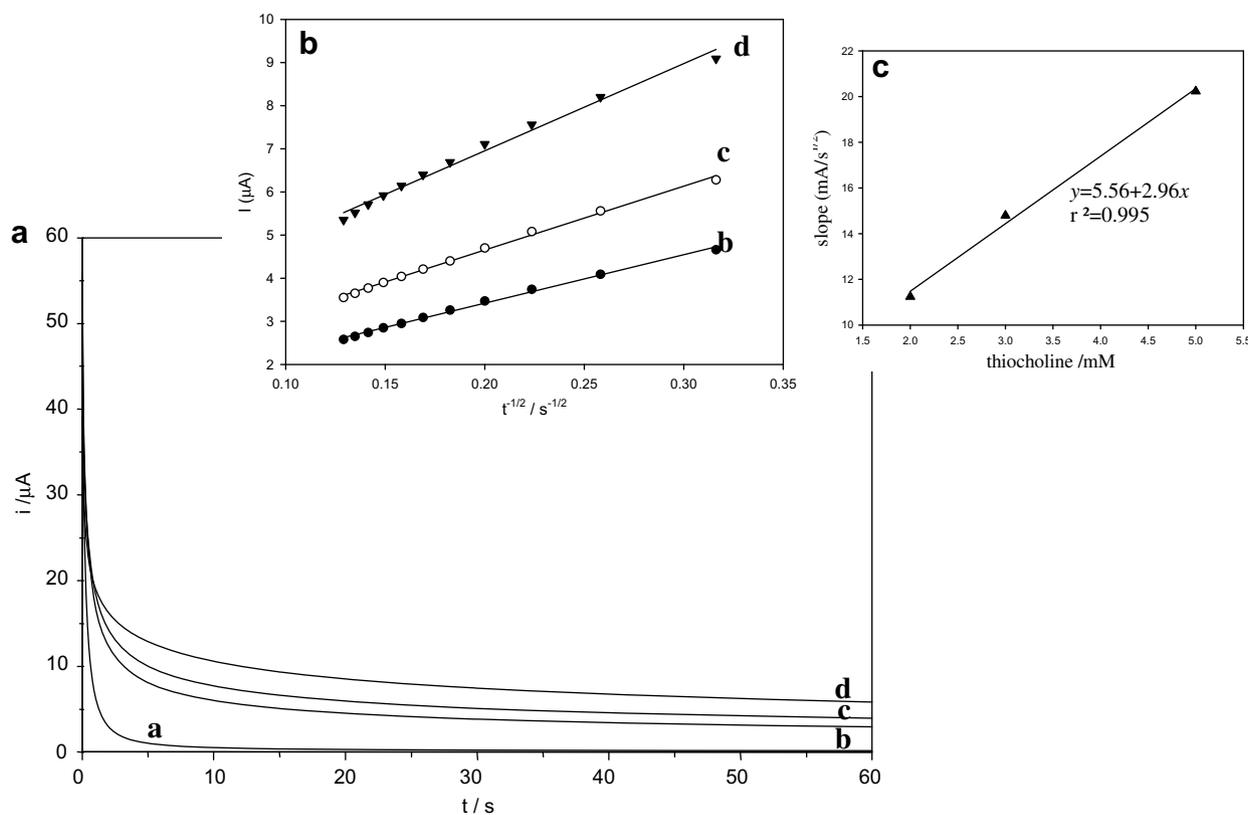
The redox semi-reaction that occurs at surface of CoHCF-SPE is the following:



The equation shows that the oxidation of thiocholine will be favoured at lower hydrogen ion concentration. Phosphate buffer (0.05 mol L<sup>-1</sup> + NaCl 0.1 mol L<sup>-1</sup>, pH = 7) was chosen to obtain high stability of the CoHCF film and a favourable thiocholine oxidation.



**Fig. 6.** Plot of  $I_{cat}/I_L$  derived from the chronoamperogram data in presence and absence of thiocholine ( $3 \text{ mmol L}^{-1}$ ) in phosphate buffer  $0.05 + \text{NaCl } 0.1 \text{ mol L}^{-1}$ ,  $\text{pH} = 7$ .



**Fig. 7.** Chronoamperograms using CoHCF-SPE in Britton–Robinson buffer + NaCl  $0.1 \text{ mol L}^{-1}$ ,  $\text{pH} = 7$  in absence (a) and in presence of thiocholine  $2$  (b),  $3$  (c), and  $5 \text{ mmol L}^{-1}$  (d) (Fig. 7a). Inset: plot of  $I$  vs.  $t^{-1/2}$  obtained from chronoamperometric experiments (Fig. 7b). Inset: plot of the slopes of linear segments taken from  $I$  vs.  $t^{-1/2}$  plot against thiocholine concentration (Fig. 7c).

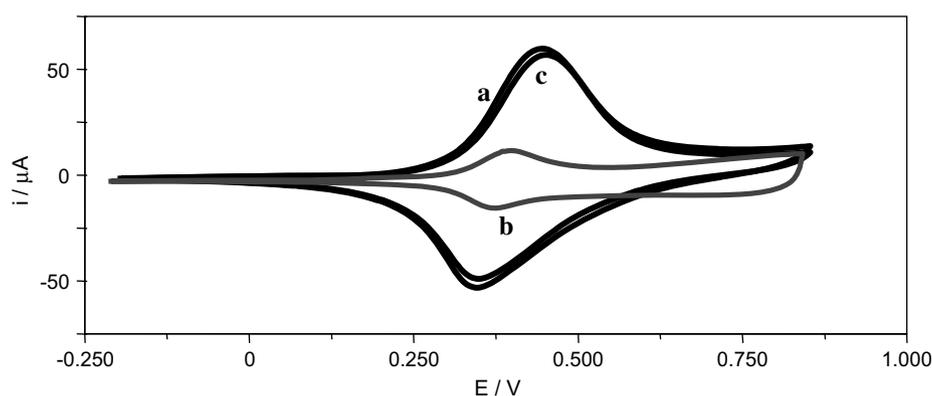
### 3.5. Thiocholine sensors

The good electroanalytical performances of the sensor developed were then confirmed in amperometric batch system; in fact our purpose is the development of sensor for thiocholine detection as a platform for an amperometric biosensor for pesticide detection.

The sensor was challenged applying a potential of  $+0.5 \text{ V}$  vs. Ag/AgCl. This potential was chosen because it corresponds to the best sensitivity (data not shown). A detection limit ( $s/n = 3$ ) of  $5 \times 10^{-7} \text{ mol L}^{-1}$  together with a linear range up to  $1 \times 10^{-5} \text{ mol L}^{-1}$  has been observed. The sensor has also demonstrated a sensitivity equal to  $435 \text{ mA mol}^{-1} \text{ L cm}^{-2}$  which is higher than other SPE-sensors reported in the literature, such as that for a SPE modified

**Table 1**  
Different response of CoHCF-SPE toward thiol compounds ( $1 \times 10^{-5}$  mol L $^{-1}$ ). Batch amperometric analysis in stirred solution, applied potential = +0.5 V vs. Ag/AgCl. Phosphate buffer 0.05 mol L $^{-1}$  + NaCl 0.1 mol L $^{-1}$ , pH 7.0.

Analyte	Chemical formula	Signal (nA)
Cysteamine (2-mercaptoethylamine)	HS-CH $_2$ -CH $_2$ -NH $_3^+$	195
Thiocholine	HS-CH $_2$ -CH $_2$ -N $^+$ -(CH $_3$ ) $_3$	105
Cysteine		69
Homocysteine		44
N-Acetylcysteine		9
Glutathione		6



**Fig. 8.** Cyclic voltammograms of the CoHCF-SPEs in phosphate buffer + NaCl pH = 7, scan rate 50 mV/s 1 h after the sensor production (a), after 1 month at RT (b) and after 1 month at RT under vacuum (c).

with cobalt phthalocyanine (24 mA mol $^{-1}$  L cm $^{-2}$ ) or Prussian Blue (143 mA mol $^{-1}$  L cm $^{-2}$ ) [17]. Moreover, the inter and intra electrode reproducibility has been evaluated by studying the response to  $5 \times 10^{-6}$  mol L $^{-1}$  thiocholine for ten successive measurements and for ten different sensors. The sensors showed an intra and inter electrode RSD% equal to 6% and 7%, respectively.

### 3.6. Interferences study

The CoHCF sensor was challenged also in amperometric batch conditions using some thiols in order to investigate (1) the possible interference from other thiols during the thiocholine measurement and (2) to compare the CoHCF behaviour toward thiols oxidation to Prussian Blue to evidence the advantage of CoHCF sensor.

The amperometric response of a series of thiols has been evaluated at the potential chosen for thiocholine (i.e. +0.5 V vs. Ag/AgCl) and the results are summarised in Table 1.

The thiocholine sensor is useful because it can be used as platform for pesticide biosensor with environmental applications. Usually at the concentration analysed the thiols tested are not present in environmental samples.

In order to develop an organophosphorous and carbammic pesticides biosensor, the cholinesterase enzyme must be used. The cho-

linesterase inhibition is an irreversible inhibition, this means to reach a low detection limit, a low enzyme concentration and a high incubation time are necessary. The irreversible inhibition permits to use a measurement procedure called "medium exchange" in which is possible to avoid the electrochemical interferences during the pesticides measurement [17]. Briefly, the enzymatic activity measurement, before inhibition, is carried out in buffer solution in presence of enzymatic substrate. After, the biosensor is made in contact with the sample contaminated with pesticides, then the biosensor is rinsed several times and the residual activity is measured in another new buffer solution in presence of enzymatic substrate and in absence of any interfering species. In this way, it is possible to avoid the electrochemical interferences and the only requirement is a sensitive thiocholine sensor.

In addition, we have made a comparison between CoHCF and Prussian Blue electrocatalytic effect towards thiols oxidation. The CoHCF behaviour is similar to Prussian Blue in fact highest reactivity at CoHCF-SPE was observed with thiols positively charged at the pH used (cysteamine, thiocholine) while thiols with a negative charge (cysteine, N-acetylcysteine and glutathione) give a slight response. However, we would like to underline that using CoHCF-SPE the signal ratio thiocholine/cysteamine (0.54) is higher than the one obtained with SPE modified with Prussian Blue (0.27)

demonstrating that the CoHCF has a better electrocatalytic property than Prussian Blue toward thiocholine oxidation.

### 3.7. Stability of the modified electrode

The storage stability of the CoHCF–SPEs was also evaluated by CV experiments. The exposure of the modified electrode to air is the most important factor which can affect the stability of the modified electrode. Electrodes stored at RT in air showed very low storage stability as shown in Fig. 8. In fact, after 1 month, a relevant decrease in the activity is observed probably due to the hydration of the film with a loss of its original structure and then of its electroactivity. To improve the storage stability, the sensor was maintained under vacuum and showed after one month an excellent storage stability (Fig. 8c).

## 4. Conclusion

A CoHCF film was chemically deposited on a SPE surface by a simple procedure. The formal potentials and the shapes of the cyclic voltammograms have shown a dependence of the electrolyte cations. The electrochemical investigations have demonstrated that the redox process was confined to the surface, confirming the immobilised state of CoHCF.

The electrocatalytic property of CoHCF toward thiocholine oxidation was for the first time demonstrated using the cyclic voltammetry technique.

The CoHCF–SPE was challenged in amperometric batch conditions at +0.5 V vs. Ag/AgCl showing for thiocholine detection a good linear range ( $5 \times 10^{-7}$ – $1 \times 10^{-5}$  mol L<sup>-1</sup>) with a low detection limit and high sensitivity equal to  $5 \times 10^{-7}$  mol L<sup>-1</sup> and 435 mA mol<sup>-1</sup> L cm<sup>-2</sup>, respectively. The good values of sensitivity and detection limit obtained showing a good analytical performance of this sensor if compared at the other mediators usually adopted for thiocholine measurement such as cobalt phthalocyanine and Prussian Blue. These findings suggest that a CoHCF–SPE can be used to assemble a sensitive ChE biosensor for pesticide detection.

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