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Electrocatalytic activity of 4-nitrophthalonitrile-modified electrode for the L-glutathione detection

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ABSTRACT

The present work describes the substantial electrocatalytic activity of $(NC)_2C_6H_3$–NHOH/$(NC)_2C_6H_3$–NO redox couple-modified electrode toward the low voltage detection of L-glutathione (GSH), in neutral medium, at an applied potential of 0.4 V versus Ag/AgCl. After optimizing the operational conditions, the sensor provided a linear response range for GSH from 8.0 up to 83.0 μmol L^{-1} with sensitivity, detection and quantification limits of 54 nA L μmol^{-1}, 2.7 μmol L^{-1} and 8.0 μmol L^{-1}, respectively. The proposed sensor presented higher sensitivity when compared to other modified electrodes described in the literature and showed a stable response for at least 100 successive determinations. The repeatability of the measurements with the same sensor and different sensors, evaluated in terms of relative standard deviation, were 4.1 and 5.0%, respectively, for $n = 10$. The developed sensor was applied for GSH determination in yeast extract and the results were statistically the same with those obtained by the comparative method described in the literature at a confidence level of 95%.

1. Introduction

Glutathione (L-γ-glutamyl-L-cysteinylglycine), in addition to being a cofactor for the GPx enzymes, is involved in many other metabolic processes, maintaining communication between cells through gap junctions and generally preventing protein–SH groups from oxidizing and cross-linking [1]. Due to its presence in high levels, in cells, it is the main nonprotein thiol involved in the antioxidant cellular defence and most abundant low molecular mass thiol found in mammals cells [2]. It is a radioprotective agent [1] and the main sulphur compound in yeasts [3]. It is a physiologically significant aqueous antioxidant, capable of scavenging reactive oxygen and nitrogen species, which are thought to contribute to the development of many common diseases including cancer, heart attack, stroke, arthritis [1,4,5].

Several methods have been available for determination of glutathione (GSH), such as high-performance liquid chromatography [6–8], spectrofluorimetry [9], spectrophotometry [10,11]. Most of them experienced difficulties with sample preparation, the necessity of molecules derivatization or the lack of sufficient sensitivity, limiting their practical utility [12]. Electrochemical methods, however, present the advantages of simplicity and high sensitivity; particularly, chemically modified electrodes are contributing decisively for this purpose. A wide variety of compounds has been incorporated to the electrode as electron transfer mediators for electrooxidation of GSH, for example, TTF-TCNQ [13], abrasive of multiwall carbon nanotubes [14], enzymes [15,16], indium hexacyanoferrate [17], Ru complexes [18] and cobalt phthalocyanine [19].

Recently, a new type of mediator was studied by us, based on 4-nitrophthalonitrile-modified carbon paste electrode [20]. The nitro compounds, in their oxidized state, have no electrocatalytic activity for thiol oxidation. However, when electrochemically reduced, the nitro-group is transformed into a hydroxylamino functionality. The resulting hydroxylamine can, thus, be oxidized reversibly to the corresponding nitroso compound (RNO/RNHOH couple), by a $2e^-/2H^+$ redox process, giving a stable $(NC)_2C_6H_3$–NHOH/$(NC)_2C_6H_3$–NO redox couple-modified electrode.

In this sense, the present work explores the electrocatalytic activity of Ar–NO/Ar–NHOH redox couple from 4-nitrophthalonitrile modified electrode for L-glutathione detection. The application of this electrode for GSH determination in yeast extract containing L-glutathione showing its reliability is also presented.

2. Experimental

2.1. Chemicals and solutions

All chemicals were of analytical grade. Graphite powder (99.9%) and mineral oil were purchased from Aldrich, Milwau-

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kee, USA. L-Glutathione (GSH) (pK_{aCOOH} = 2.126, pK_{aCOOH} = 3.512, pK_{aSH} = 8.736, $pK_{aNH^{3+}}$ = 9.655) [21], McIlvaine (Na_2HPO_4 and citric acid), piperazine-*N*-*N*-bis[2-ethanesulfonic acid] (PIPES), *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) (HEPES) and [tris(hydroxymethyl)aminomethane], 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), L-(+)-cysteine, L-(+)-ascorbic acid, L-(+)-glutamic acid, glycine and glucose were acquired from Sigma, St. Louis, USA. Disodium, monosodium phosphate (Na_2HPO_4 and NaH_2PO_4) and Na_2H_2EDTA were acquired from Synth, São Paulo, Brazil. 4-Nitrophthalonitrile was synthesized by the method described by Young and Onyebuago [22]. The solutions were prepared by using water purified in a Milli-Q Millipore system and the pH values of the buffer solutions were determined with a Corning pH/Ion Analyser model 350. Ellman's reagent DTNB, utilized as a reference for the GSH determination, was prepared at a concentration of 3 mmol L^{-1} in 50 mmol L^{-1} TRIS buffer containing 3 mmol L^{-1} Na_2H_2EDTA, adjusting the solution pH (8.0) with HCl. Working standard solutions were prepared daily by appropriate dilution of the stock solutions with deionized water.

2.2. Construction of the sensors

The modified carbon paste was prepared by mixing 35 mg of graphite powder with 5 mg of 4-nitrophthalonitrile (0.029 mmol) and 20 μL of mineral oil, added to give consistence to the paste. The influence of the ratio between 4-nitrophthalonitrile and graphite powder used in the modified electrode preparation on the peak current was investigated in the 1:13, 1:7, 1:3, 1:1 and 3:1 (w/w) proportion. This paste was put into a cavity in the extremity of a Teflon® tube containing a graphite rod, for the electrical contact with the paste. To activate the surface of the modified electrode, initially the electroactive species, the polynitrile hydroxylamine, was electrogenerated *in situ* from the nitrophthalonitrile, after cycling in the potential range between −0.5 and 1.0 versus Ag/AgCl in the first scan in 0.1 mol L^{-1} phosphate buffer (pH 7.0), with a scan rate of 0.02 V s^{-1}, directly at the modified carbon paste electrode and it was characterized by cyclic voltammetry as recently showed by Kubota and coworkers [20].

All the responses obtained with the proposed sensor were given in terms of current density. Thus, the geometric area of the working electrode (sensor) was determined as ($A = \pi r^2$) and it presented the value of 0.2 cm^2.

2.3. Electrochemical and spectrophotometric measurements

The voltammetric and amperometric measurements were carried out with a potentiostat PGSTAT-30 Model from Autolab Echo Chemie (Utrecht, The Netherlands) connected to a PC (Software GPES 4.9). An electrochemical cell with three electrodes was used with a Ag/AgCl (saturated KCl) electrode as reference, a Pt wire as auxiliary and unmodified or 4-nitrophthalonitrile-modified carbon pastes as working electrodes were used for all measurements. The measurements were carried out, using 5.00 mL of buffer solutions. Oxygen was removed by bubbling nitrogen through the solution.

The spectrophotometric measurements were performed in a Pharmacia Biotech® Ultrospec 2000 model spectrophotometer, connected to a microcomputer (software Wavescan®), using a quartz cuvette with 1.0 cm optical path. The solution pH was measured employing a pH electrode connected to a pH-meter (Corning pH/Ion Analyzer 350, Corning, NY).

2.4. Procedure for the sample preparation for amperometric and spectrophotometric determinations

For amperometric analysis, the yeast extract samples (Biorigin) were prepared by dissolving the sample (350 mg) in 5.00 mL of 0.1 mol L^{-1} HCl and then the solution was centrifugated (1000 × *g*, 10 min, 25 °C). After this step, an aliquot of 100 μL of the supernatant prepared centrifugating the solution was added to the cell containing 5.00 mL of the supporting electrolyte to be measured.

For the spectrophotometric measurements, an aliquot of 50 μL of the supernatant yeast extract were mixed with 2.45 mL DTNB. Other steps were performed according to already described procedure [23], which is based on the reaction of glutathione and DTNB (Ellman's reagent), generating 2-nitro-5-mercapto-benzoic acid (TNB). This was spectrophotometrically monitored at 412 nm.

3. Results and discussion

3.1. Activated 4-nitrophthalonitrile modified electrode and electrocatalytic oxidation of GSH

Fig. 1 shows the cyclic voltammograms recorded in 0.1 mol L^{-1} phosphate buffer at pH 7.0 for the activated 4-nitrophthalonitrile-modified electrode in presence (a) and in absence of GSH (b). For comparison purpose, cyclic voltammograms obtained with an unmodified electrode in the presence (c) and absence of GSH (d) are also presented. In these voltammograms (Fig. 1c and d), there is no evidence of peaks, indicating that the direct oxidation of GSH is out of these limits (E_{pa} > 0.5 V versus Ag/AgCl). In Fig. 1b, the redox system I_a/I_c, relative to the $(NC)_2C_6H_3$–NHOH/$(NC)_2C_6H_3$–NO redox couple is observed at potentials of E_{pIa} = 0.10 V and E_{pIc} = 0.05 V versus Ag/AgCl. After adding GSH to the solution, wave I_a (oxidation peak of the 4-hydroxylaminephthalonitrile) is still present, with a slight increase and a second wave (II_a) appears, with a comparatively higher peak current, at a potential of 0.350 V versus Ag/AgCl. On the reverse scan, wave I_c is no longer observed. These facts suggest that GSH/GS^- ($pK_{aNH^{3+}}$ = 9.65) reacts with the electrogenerated nitrosophthalonitrile ($(NC)_2C_6H_3$–NO), forming adducts, which are oxidizable species, as shown before by Komiyama and Fujimori in biological media [24] and Kubota and coworkers [20]. The adduct is, then, dissociated in ($(NC)_2C_6H_3$–NO) and 1/2(GSSG) [20]. The nitroso compound ($(NC)_2C_6H_3$–NO) can react again with

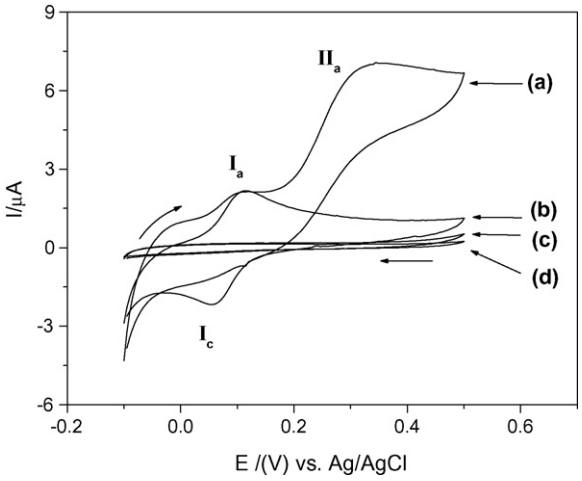


Fig. 1. Cyclic voltammograms for an activated 4-nitrophthalonitrile modified electrode, in the presence (a) and absence (b) of 0.2 mmol L^{-1} GSH; unmodified carbon paste electrode in the presence (c) and absence (d) of 0.2 mmol L^{-1} GSH, in phosphate buffer solution at pH 7. Scan rate: 0.05 V s^{-1}.

new GSH in a new cycle of the reaction increasing the intensity of oxidation peak current, which is several times greater than that for GSH without catalyst on the electrode surface. A definite characterization of the formed adduct is out of the scope of the present paper.

3.2. Influence of the amount of 4-nitrophthalonitrile on carbon paste electrode in the sensor response

The influence of the ratio of 4-nitrophthalonitrile and graphite powder used in the modified electrode preparation on the peak current was investigated in the proportions of 1:13, 1:7, 1:3, 1:1 and 3:1 (w/w), in phosphate buffer solution (pH 7.0) containing 60.0 μmol L^{-1} of GSH, with an oxidation potential of 0.35 V versus Ag/AgCl. The results indicated that the best analytical signal was obtained using a proportion 1:7 (1.60 μA) and therefore was chosen for further experiments. In ratios higher than 1:7, good homogenization of the carbon paste was not obtained, and for lower ratios, the current was smaller.

3.3. Influences of the solution, pH, buffer nature and concentration

The influence of the solution pH in the electrochemical response in the presence GSH (60 μmol L^{-1}) using 0.1 mol L^{-1} phosphate buffer at pH 6.0, 6.5, 7.0, 7.5 and 8.0 was studied. In order to optimize the electrocatalytic response of the modified carbon paste electrode towards GSH oxidation, the effect of pH on the catalytic oxidation behavior was also investigated (Fig. 2a and b). Thus, the peak current was investigated in the range from pH 6.0 up to 8.0. At pH 7.0, the peak current gives a maximum. A decrease in the current is observed when the solution pH is higher than 7.0.

A possible explanation for the optimal pH at 7.0 is structural: the electronic density increases, facilitating the oxidation of the adduct formed in the chemical step. In pH values >7.0, two effects could be operating: (i) lower amount of the protonated species and (ii) lower amount of the mediator on the carbon paste surface [20], which contribute to a lower peak current. Thus, the optimum pH for further studies was set in 7.0 as shows Fig. 2a. A linear correlation obtained for E_p versus pH with a slope of 0.06 V/pH (Fig. 2b) from pH 6.0 up to 8.0, is close to that expected for an electrodic reaction with the ratio e^-/H^+ equal to one (0.0592 (n_p/n_e) V/pH, where $n_p = n_e$ at 25 °C [25]. Thus, the number of protons involved in this process should be the same of the electrons.

The electrocatalytic activity of $(NC)_2C_6H_3$–NHOH/$(NC)_2C_6H_3$–NO modified carbon paste electrode for the L-glutathione detection does not only depend on pH, but also on the buffer solution and its concentration, which can change the activity or the stability of the sensor. So, the influence of the buffer solution on the sensor response was also tested in four different buffer solutions (HEPES, PIPES, Macllvaine, TRIS and phosphate) with concentrations of 0.1 mol L^{-1}, pH 7.0 and indicated that phosphate buffer solutions give the best responses, probably due to the facility of phosphate anions to diffuse through the carbon paste electrode in comparison with the ions of HEPES, PIPES, Macllvaine and TRIS buffers. In this sense, the phosphate buffer solution was chosen.

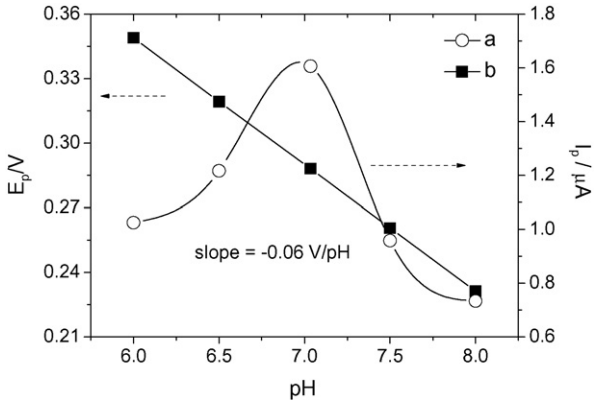


Fig. 2. Influence of the solution pH on the peak current (a), peak potentials (b) obtained by CV in 60.0 μmol L^{-1} GSH. Measurements carried out in 0.1 mol L^{-1} phosphate buffer solution at pH 7.0.

Furthermore, the influence of the phosphate buffer concentration was carried out in different concentrations of phosphate (0.025, 0.05, 0.10, 0.20 and 0.25 mol L^{-1}). Phosphate buffer concentrations from 0.1 up to 0.25 mol L^{-1} presented almost constant current and the best response was obtained with 0.1 mol L^{-1} phosphate buffer solution. In [PBS] values <0.1 mol L^{-1}, it is noticeable that the electron transfer slows down, fact that can be attributed to the lack of charge transport to keep the electroneutrality of the electrode. In this sense, the concentration of 0.1 mol L^{-1} was chosen for further experiments.

3.4. Electrochemical studies of the GSH oxidation on modified electrode

More detailed studies of peak current were performed by cyclic voltammetry in 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) to obtain new insights into the GSH electrocatalytic oxidation on the sensor surface. Firstly, a plot of the catalytic current I_p versus the square root of the potential scan rate ($v^{1/2}$) in the rate from 0.01 up to 0.450 V s^{-1} was built and resulted in a straight line (data not shown), expressed by $I_p\,(\mu A) = 0.3 + 27.9v^{1/2}(V\,s^{-1})$. It suggests that at a sufficiently large overpotential, the reaction is controlled by mass-transport in the studied scan rate range as well as is under catalytic control in low scan rates (positive intercept). Thus, these results show that the overall electrochemical oxidation of GSH at modified electrode is controlled by the cross-exchange process between GSH and the redox site of the $(NC)_2C_6H_3$–NHOH/$(NC)_2C_6H_3$–NO and diffusion of GSH. Using the angular coefficient of this expression, it is possible to determine the number of electrons (n) involved in the GSH oxidation at the modified electrode. As can be seen in Fig. 1, the cyclic voltammetric experiments of GSH on 4-hydroxylaminephthalonitrile-modified carbon paste electrode presents one major anodic peak in the investigated potential range. Thus, assuming an irreversible oxidation of GSH on the modified electrode the following equation was used [26]:

$$I_p = (2.99 \times 10^5)n[(1-\alpha)n_a]^{1/2}C_o^* A D^{1/2} v^{1/2} \quad (1)$$

where I_p is the peak current, n the number of total electrons involved in the reaction, α the electron transfer coefficient, n_a represents the number of electrons involved in the rate-determining step, "D_o" (cm^2 s^{-1}) the diffusion coefficient of the electroactive species, C_o^* (mol cm^{-3}) the concentration of the electroactive species and v is the potential scan rate. The value of the concentration and diffusion coefficient used for GSH in aqueous solution were 0.2 mmol L^{-1} (2×10^{-7} mol cm^{-3}) and 6.47×10^{-6} cm^2 s^{-1} [2,27], respectively.

Considering the necessary knowledge of the $[(1-\alpha)n_a]$ value presented in Eq. (1), in addition considering that the $(1-\alpha)n_a$ value has to be known, one approach was employed for the glutathione oxidation reaction, based on the shift of the peak potential as a function of scan rate [26]. Thus, the dependence of the position of the peak potential with the potential scan rate (E_{pa} versus log v) and resulted in a regression equation represented by $E_p(V) = 0.37 + 0.05 \log [v\,(V\,s^{-1})]$ for scan rates between 0.030 and

0.400 V s^{-1}. In this sense, based on the simplified expression for an irreversible reaction, the change in E_{pa} for each 10-fold increase in v is $1.15RT[(1-\alpha)n_a]F$ [26], the plot of E_{pa} versus $\log v$ indicates a linear variation with slope ($\Delta E_{pa}/\Delta \log v$) found to be 0.05 V, the values of $[(1-\alpha)n_a]$ was calculated as being 0.6. Thus, using this value in Eq. (1) and the slope 27.9 μA/(V s^{-1})$^{1/2}$ extracted from plot I_p versus $v^{1/2}$, the value of n was calculated to be 1.18, suggesting a transfer mechanism of 1.0 electron for the electrocatalytic oxidation of GSH. This result is in agreement to the works reported in the literature based on the catalytic oxidation of GSH [2]. If the number of electrons determined in the GSH oxidation in this work was 1.0 as well as considering the behavior of the peak potential with the solution pH, there is only one possibility for pH between 6.0 and 8.0. The number of protons involved in this process should be equal to the number of eletrons, or 0.059 (n_p/n_e) V/pH where $n_p = n_e$ (Fig. 2b).

3.5. The sensor characteristics

For amperometric measurements the applied potential has an important influence over the sensor response, because the applied potential contributes to the sensitivity of the system. Thus, the characteristics of the activated 4-nitrophthalonitrile modified carbon paste electrode were verified by amperometric experiments and an initial study was performed in order to determine the best potential to be applied on the electrode. In this sense, the applied potential was chosen based on the measurements of the catalytic current intensities in the optimized conditions and the highest current was verified at an applied potential of 0.4 V versus Ag/AgCl.

In order to obtain an analytical curve for the developed sensor, amperograms for GSH oxidation were carried out at different concentrations in 0.1 mol L^{-1} phosphate buffer at pH 7.0, after optimizing the experimental parameters (Fig. 3a). The proposed sensor showed a linear response range from 8.0 up to 83.0 μmol L^{-1} (Fig. 3b), which can be expressed according to the following equation:

$$\Delta I\,(\mu A) = 0.210(\pm 0.040) + 0.054(\pm 0.001)\,[GSH]\,(\mu mol\,L^{-1}) \quad (2)$$

with a correlation coefficient of 0.998 (for $n = 10$) with better sensitivity and detection limit than those reported in the literature [13,18,28–34]. Such good sensitivity of 54 nA L μmol^{-1} can be attributed to the efficiency of the electron transfer between the 4-nitrosophthalonitrile and GSH [20]. A detection limit of 2.7 μmol L^{-1} was determined using a 3σ/slope ratio and quantification limit was 8.0 μmol L^{-1} using 10σ/slope, where s is the standard deviation of the mean value for 10 amperograms of the blank, determined according to the IUPAC recommendations [35].

The sensor response time was very short, reaching 95% of its maximum response in 0.1 s as observed in insert of Fig. 3a, which also shows the high stability of the signal as a function of time. This response time is excellent considering that it is a carbon paste electrode. Probably the design and procedure used to construct the electrode, packed it so well that it become difficult for the solution to diffuse through the paste and this may contribute to its behavior.

Table 1 lists the reported works involving modified electrodes with several mediators for GSH determination, for a comparison purpose. According to Table 1, it can be noted that few articles utilize neutral medium for GSH determination. Besides, the oxidation potentials for GSH are relatively high when compared to the present work, and the detection limit and sensitivity of the present work are also better (Table 1) [29,32–34]. Such good sensitivity can be attributed to the efficiency of the electron transfer between 4-nitrosophthalonitrile of the modified mediator to the sulfhydryl group GSH.

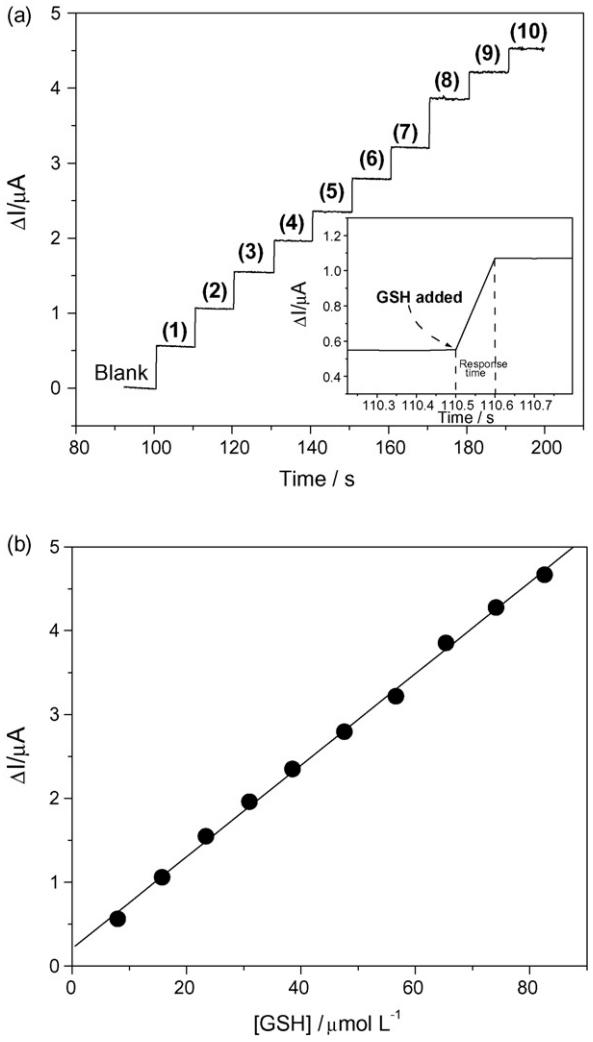


Fig. 3. (a) Amperometric measurements for the electrooxidation of GSH on the activated 4-nitrophthalonitrile modified electrode obtained in 0.1 mol L^{-1} phosphate buffer at pH 7.0 at concentration: (1) 8.0 to (10) 83.0 μmol L^{-1} and (b) the calibration plot. Applied potential of 0.4 V vs. Ag/AgCl and 0.1 mol L^{-1} phosphate buffer at pH 7.0.

3.6. Effects of interferences

Study of interferences on the electrode response is useful to set up the sample preparation with the goal to minimize their effects. In this work, the interferences were considered to be the compounds that are structurally related to GSH and present in yeast and blood samples, since this electrode was developed to analyze GSH in yeast or biological samples. For interference studies, the signal for a c_{GSH} of 50 μmol L^{-1} was recorded and the obtained signal was compared to those of the mixture of GSH and interfering compound in the ratio of 5:1. Such ratio was chosen since these interferences are not found in yeast and blood at higher concentrations [3,33]. The obtained results can be observed in Table 2. These results showed a significant interference from ascorbic acid and cysteine. However, glycine, glutamic acid and glucose have no interference on the electrode response. Although ascorbic acid and cysteine show interference, they are not present in yeast samples and at a low level in the blood samples. Moreover, the interference from ascorbic acid can be minimized by using ascorbate oxidase enzyme which exhibits high selectivity to oxidation of ascorbic acid [36,37].

Table 1
Experimental conditions and analytical parameters for GSH determination

Electrode	Method	E_p (V)	Electrolyte	LOD (μmol L^{-1})	Linear range (μmol L^{-1})	Sensitivity (nA L μmol^{-1})
Sensor based on TTF-TCNQ [13]	Amperometry	0.200 vs. SCE (⇑)	0.1 mol L^{-1} Phosphate buffer containing 0.1 mol L^{-1} KCl and 0.5 mmol L^{-1} $Na_2H_2EDTA{\cdot}2H_2O$ (pH 8.0)	0.3 (⇑)	5.0–340.0 (⇑)	11.0 (⇓)
Carbon ceramic electrodes modified with a Ru-complex [18]	Amperometry	0.800 vs. Ag/AgCl (⇓)	0.1 mol L^{-1} Phosphate buffer (pH 2.0)	1.0 (⇑)	5.0–700.0 (⇑)	7.8 (⇓)
Pd-IrO_2 modified electrode [28]	Amperometry with HPLC	0.850 vs. Ag/AgCl (⇓)	0.1 mol L^{-1} PBS solution (pH 3.0) containing 1.0 10^{-4} mol L^{-1} Na_2EDTA	2.0 (⇑)	10.0–800.0 (⇑)	–
Nitroso phenyl modified carbon [29]	SWV	−0.100 vs. SCE (⇑)	0.05 Phosphate buffer (pH 7.0)	8.1 (⇓)	–	51.0 (⇓)
Electrochemical detection of thiols in biological media [30]	CV	0.200 vs. SCE (⇑)	Tissue culture media (pH 7.0)	1.0 (⇑)	6.0–59.0 (⇓)	–
Edge plane pyrolytic graphite electrode [31]	CV	0.650 vs. SCE (⇓)	0.1 mol L^{-1} phosphate buffer (pH 7.0)	2.7 (⇔)	10.0–80.0 (⇔)	39.0 (⇓)
Boron-doped diamond electrode [32]	Chronoamperometry	0.850 vs. SCE (⇓)	0.1 mol L^{-1} phosphate buffer (pH 7.5)	5.8 (⇓)	10.0–100.0 (⇔)	22.9 (⇓)
Ruthenium(III)diphenyldithiocarbamate modified carbon paste electrode [33]	Amperometry	0.360 vs. SCE (⇔)	0.1 mol L^{-1} KNO_3 (pH 3.0)	15.2 (⇓)	–	–
Biosensor based on glutathione peroxidase immobilized in a carbodiimide [34]	Amperometry	0.650 vs. SCE (⇓)	0.1 mol L^{-1} phosphate buffer (pH 7.8) and 0.1 mol L^{-1} KCl	–	20.0–140.0 (⇓)	2.45 (⇓)
Sensor based on $(NC)_2C_6H_3$–NHOH/$(NC)_2C_6H_3$–NO redox couple from 4-nitrophthalonitrile-modified electrode (this work)	Amperometry	0.400 vs. Ag/AgCl	0.1 mol L^{-1} phosphate buffer (pH 7.0)	2.7	8.0–83.0	54.0

CV, cyclic voltammetry; HPLC, high performance liquid chromatography; SWV, square wave voltammetry. Ag/AgCl vs. SCE = −0.045 V. The qualitative criteria adopted were: better (⇑), near (⇔) or worst (⇓) than the sensor based on this work, took as reference.

3.7. Stability of 4-nitrophthalonitrile on the carbon paste

The stability of the carbon paste electrode modified with activated 4-nitrophthalonitrile was checked in presence of GSH, performing successive cyclic voltammograms in a potential range between −0.1 and 0.5 V versus Ag/AgCl. After 100 determinations, no significant change was observed in the voltammetric response, as well as for the electrode stored at room temperature, for 1 month.

The modified electrode presents a good repeatability for GSH determination. The relative standard deviation (R.S.D.) for 10 determinations of 60.0 μmol L^{-1} GSH was 4.1%. Additionally, a series of 20 sensors prepared in the same manner and tested at phosphate buffer (pH 7.0) containing 60.0 μmol L^{-1} GSH gives responses with a relative standard deviation lower that 5.0%, indicating a good stability and repeatability probably due to π–π interactions that should favor the adsorption in the matrix, leading to fairly stable in its voltammetric responses [20].

3.8. Determination of GSH in yeast samples

The proposed method was applied for determination of GSH in three yeast samples in triplicate. The concentrations of GSH in the yeast samples were determined using the standard addition method and these results were compared with the spectrophotometric method and good agreement was obtained (Table 3). Another interesting point to be emphasized is that a systematic error should exist since the spectrophotometric method requires a considerable time for sample preparation in relation to the electrochemical method. This occurs owing to the loss of the analyte during the preparation of the samples for the spectrophotometric analysis, as described by Calvo-Marzal et al. [13]. On the other hand, the agreement between the results obtained by the proposed and reference methods was evaluated through the paired Student's *t*-test and it was possible to observe that, at the 95% confidence level, there was no statistical difference between the comparative and the proposed methods. It is important to indicate that, although there was no serious interference from the matrix, the standard addition method was used to confirm the values obtained by the analytical curve method owing to the complexity of the sample.

Table 3
GSH determination in three yeast samples in triplicate

Samples	Proposed method (w/mg)	Comparative method (w/mg)
A	1.87 (±0.03)	1.86 (±0.01)
B	1.81 (±0.01)	1.80 (±0.08)
C	1.83 (±0.03)	1.82 (±0.05)

w: mass obtained in 1000 mg yeast sample.

3.9. Recovery tests for the proposed method

For an additional check on the accuracy of the proposed method and possible matrix interferences, analytical recovery experiments

Table 2
Recovery values (%) obtained for 50 μmol L^{-1} GSH in the presence of interfering compounds of 10 μmol L^{-1}

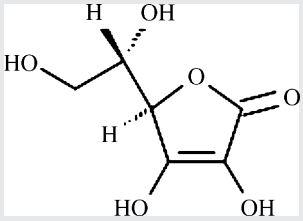
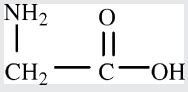
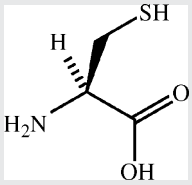
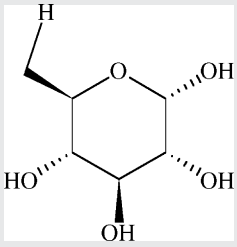
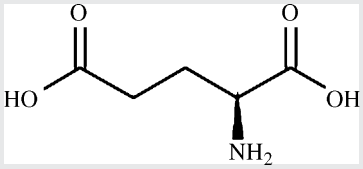
Interfering structure compounds		GSH added (μmol L^{-1})	GSH found (μmol L^{-1})	Recovery (%)
L-(+)-Ascorbic acid		50	55.5 ± 0.2	111 ± 1
Glycine		50	49.3 ± 0.1	99 ± 1
L-(+)-Cysteine		50	52.9 ± 0.3	106 ± 2
D-(+)-Glucose		50	50.6 ± 0.3	101 ± 1
L-(+)-Glutamic acid		50	50.5 ± 0.2	101 ± 1

Table 4
Recovery data of GSH in three samples of yeast ($n = 3$) obtained with the modified electrode

Samples	GSH added ($\mu mol\,L^{-1}$)	GSH expected ($\mu mol\,L^{-1}$)	GSH found ($\mu mol\,L^{-1}$)	Recovery (%)
A	0.0	–	8.35 (±0.02)	
	8.50	16.85	16.82 (±0.06)	99.8 (±0.2)
B	0.0	–	8.08 (±0.05)	
	8.50	16.58	16.50 (±0.09)	99.5 (±0.1)
C	0.0	–	8.17 (±0.05)	
	8.50	16.67	16.70 (±0.09)	100.2 (±0.3)

were performed by adding known amounts of GSH to three samples of yeast. The percentages of recovery were calculated by comparing the concentration obtained from the samples with actual and added concentrations. The recoveries for the samples are shown in Table 4. It can be clearly observed that there is no influence of the matrices on the sensor for the evaluated samples.

4. Conclusions

These studies demonstrate that electrochemically activated carbon paste electrode modified with 4-nitrophthalonitrile, that leads to reversible $(NC)_2C_6H_3$–NHOH/$(NC)_2C_6H_3$–NO redox couples on the surface is a feasible alternative for the analytical determination of glutathione in yeast sample at low overpotentials and neutral medium. Thus, optimization of the experimental conditions for amperometry allowed a good detection limit and sensitivity, of $2.7\,\mu mol\,L^{-1}$ and $54\,nA\,L\,\mu mol^{-1}$, respectively for GSH determination. Moreover, a qualitative criterion was adopted to facilitate the comparison of the results obtained in this work with similar reports in the literature: better (⇑), near (⇔) or worst (⇓) than the present sensor taken as a reference. In Table 1 are summarized the results for each investigated sensor/parameter, as well as the final attributed value, the sensitivity taken as the main advantage.

This work demonstrated that the present modified carbon paste electrode is a sensitive, robust and stable sensor showing great potential for GSH determination. It opens the way for other important applications and good promise such as the study of thiols in biological fluids, particularly in blood samples.

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