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ORIGINAL ARTICLE

Simultaneous electrochemical determination of L-cysteine and L-cysteine disulfide at carbon ionic liquid electrode

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Abstract A linear sweep voltammetric method is used for direct simultaneous determination of L-cysteine and L-cysteine disulfide (cystine) based on carbon ionic liquid electrode. With carbon ionic liquid electrode as a high performance electrode, two oxidation peaks for L-cysteine (0.62 V) and L-cysteine disulfide (1.3 V) were observed with a significant separation of about 680 mV (vs. Ag/AgCl) in phosphate buffer solution (pH 6.0). The linear ranges were obtained as 1.0–450 and 5.0–700 μM and detection limits were estimated to be 0.298 and 4.258 μM for L-cysteine and L-cysteine disulfide, respectively. This composite electrode was applied for simultaneous determination of L-cysteine and L-cysteine disulfide in two real samples, artificial urine and nutrient broth. Satisfactory results were obtained which clearly indicate the applicability of the proposed electrode for simultaneous determination of these compounds in complex matrices.

Keywords Carbon ionic liquid electrode · L-Cysteine · L-Cysteine disulfide · Simultaneous determination

Introduction

The ratio of thiols/disulfides, known as thiol redox state (TRS), is a critical parameter associated with various essential biochemical processes (Chen et al. 2008). This ratio is influenced by oxidative stress in body fluids. Determination of TRS is useful in diagnosis of several diseases such as hepatic cystinuria, inflammation, cardiovascular disease, neurodegenerative disease, diabetes, etc. (Chen et al. 2008; Kargosha et al. 2008; Guan et al. 2003; Gill et al. 2010). Therefore, simultaneous determination of thiols and their corresponding disulfides has become extremely important. One of the most important low molecular weight thiol/disulfide couple is L-cysteine/L-cysteine disulfide (L-cystine), which exists in a variety of samples such as body fluids, food products, biological tissues and medicines (Kargosha et al. 2008).

In the biopharmaceutical industry, therapeutic proteins are produced by microorganisms which are grown in cell culture media. These media ideally provide all of the nutritional requirements for cell growth which lead to maximum production of the target proteins (Alwael et al. 2010). Cell culture media can be divided into two main types; complex media and chemically defined media. Complex media are a source of amino acids, peptides, carbohydrates, vitamins and other minerals. Chemically defined medium is made of chemically defined ingredients of known purity added to purified water. This medium often contains mixtures of vitamins and amino acids. Temporal stability of its components is an essential parameter for high-yield production of therapeutic proteins (Alwael et al. 2010). Chemically defined media have a number of advantages over complex media, such as improved reproducibility, simplified downstream processing and analysis of final products (Alwael et al. 2010). L-Cysteine (CSH) is one of the important amino acids in chemically defined media. Since CSH can be easily oxidized to L-cysteine disulfide (CSSC) or cysteine, therefore, the ratio of CSH/CSSC demonstrates quality assay for these media (Alwael et al. 2010).

Different analytical methods have been used for simultaneous determination of CSH and CSSC such as capillary electrophoresis (Zhong and Lunte 1998), vapor phase

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Fourier transform infrared spectrometry (Kargosha et al. 2008) and liquid chromatography with different detectors (Alwael et al. 2010; Seiwert and Karst 2007; Johnson et al. 2008). These methods are time consuming and expensive. Thus, a simple, sensitive, rapid and cost-effective method is required for simultaneous determination of these two important biological compounds. Electrochemical techniques have been paid much attention due to their rapidity, convenience, low cost, high sensitivity and ease of miniaturization for small volume samples. Several electrochemical methods have been previously reported for separate determination of CSH or CSSC (Tabeshnia et al. 2010; Van Den Berg et al. 1988; Banica et al. 1994). However, the main problem of applied electrodes is the low resolution of CSH and CSSC voltammetric peaks that limits simultaneous determination of these compounds in the biological samples.

Carbon ionic liquid electrode (CILE) was introduced in 2006 for the first time as a new and high performance carbon composite electrode (Maleki et al. 2006). The main idea for fabrication of this new electrode was the replacement of conventional nonconductive organic binders in carbon paste electrodes (CPEs) with a pyridinium-based ionic liquid. Some interesting features of CILE include wide potential window in aqueous solutions, low background current, renewable surface, resistivity toward biomolecules fouling and a rapid electron transfer. It is proved that CILE can be appropriate for determination of different analytes such as L-cysteine, dopamine, ascorbic acid, uric acid, riboflavin, sulfite, etc. (Maleki et al. 2007; Safavi et al. 2006, 2010, 2008).

In this work, CILE was used as an efficient electrode for simultaneous determination of CSH and CSSC. CILE can successfully resolve the CSH and CSSC oxidation peaks without any further modification of the electrode surface. To the best of our knowledge, this is the first report on simultaneous determination of these two biological species with an electrochemical method.

Experimental

Apparatus

The electrochemical measurements were performed using an Autolab electrochemical system (Eco-Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES softwares (Eco-Chemie). The electrochemical cell was assembled with a conventional three electrode system: an Ag/AgCl/KCl (3 M) reference electrode (Metrohm) and a platinum disk as an auxiliary electrode. Different working electrodes including CILE (1.8 mm diameter), carbon paste electrode (CPE), and glassy carbon electrode (GCE, 2 mm diameter from Metrohm) were used. 0.1 M phosphate buffer solution (PBS, pH 6.0) was used as the supporting electrolyte in all of the experiments. The pH measurements were carried out using a Metrohm pH meter (model 780) with a combined pH glass electrode calibrated against standard buffer solutions at pH 4.0 and 7.0.

Reagents

L-CSH was supplied by Riedel-deHaen. L-CSSC, pyridine, diethyl ether, 1-iodooctane, nutrient broth (NB), KH_2PO_4 and Na_2HPO_4 were obtained from Merck. Ammonium hexafluorophosphate and graphite powder (particle size <100 μm) were purchased from Fluka. Deionized distilled water was used to prepare all of the solutions.

The ionic liquid (IL), octylpyridinum iodide was synthesized as previously reported (Fisicaro et al. 1996; Maleki et al. 2006). *n*-Octylpyridinum hexafluorophosphate (OPFP) was obtained by anion exchange of octylpyridinum iodide with ammonium hexafluorophosphate (Maleki et al. 2006).

Buffer solution of pH 6 was prepared from 0.1 M KH_2PO_4 and Na_2HPO_4 and used as supporting electrolyte.

Electrode preparation

The electrode preparation has been described in our previous work (Safavi et al. 2006). Briefly, the graphite powder and OPFP with a ratio of 50/50 (w/w) were hand mixed and the resulting paste was packed into the cavity (1.8 mm diameter) of a Teflon holder. To have a better uniformity in the composite and lower background current, the electrode was heated in an oven to a temperature higher than the melting point of OPFP (mp 65 °C). It was then left to cool to room temperature. An electrical contact was established via a stainless steel handle. The electrode surface was simply renewed by rubbing on a smooth paper. CPE was prepared by hand mixing of graphite powder and paraffin oil with a ratio of 70/30 (w/w).

Results and discussion

Cyclic voltammetry of CSH and CSSC at CILE

The electrochemical behaviors of CSH and CSSC were investigated by cyclic voltammetry in PBS (pH 6); that is a buffer solution commonly used in biological research (Sunitha et al. 2012). Cyclic voltammograms of CSH and CSSC in 0.1 M PBS (pH 6.0) at CILE are shown in Fig. 1. The obtained background current of CILE shows no redox peak in the applied potential window (Fig. 1a). Under electro-oxidation, CSH in the reduced form is converted to

its oxidized form (CSSC) and CSSC is oxidized to cysteic acid (CSO_3H) by the following equations (Safavi et al. 2009; Ralph et al. 1994; Sanchez-Cano et al. 1991):

$$2CSH \rightarrow CSSC + 2H^+ + 2e^- \quad (1)$$

$$CSSC + 6H_2O \rightarrow 2CSO_3H + 10H^+ + 10e^- \quad (2)$$

The oxidation peak of CSSC was observed at 1.3 V (Fig. 1b). CSH shows two electro-oxidation peaks at 0.62 and 1.3 V, corresponding to CSH oxidation and its disulfide product, respectively (Fig. 1c). Therefore, in a mixture containing both CSH and CSSC (Fig. 1d), simultaneous determination of these two species is possible by subtraction of the CSSC contribution produced by Eq. 1 from the total amount of CSSC. The experimental trend and data analysis in this work are similar to what previously reported for simultaneous determination of glutathione and glutathione disulfide (Safavi et al. 2009). Figure 1 also shows that the oxidations of CSH and CSSC at CILE are electrochemically irreversible processes within the applied potential range (0.1 to 1.5 V).

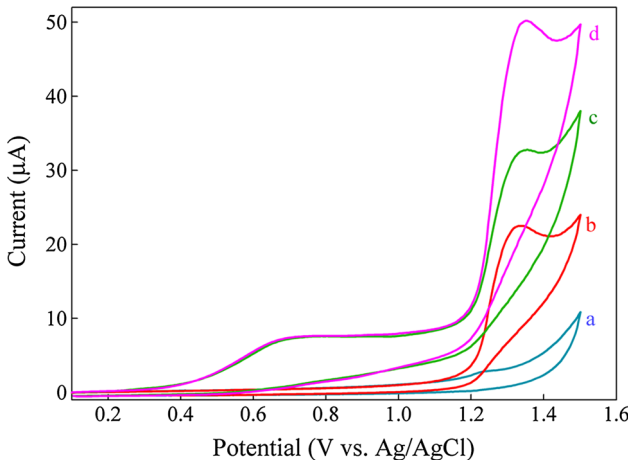

Fig. 1 Cyclic voltammograms of **a** PBS (pH 6.0) as electrolyte solution, **b** 0.5 mM CSSC, **c** 2.0 mM CSH and **d** a mixture containing 0.5 mM CSSC and 2.0 mM CSH at CILE, Scan rate 50 mV s^{-1}

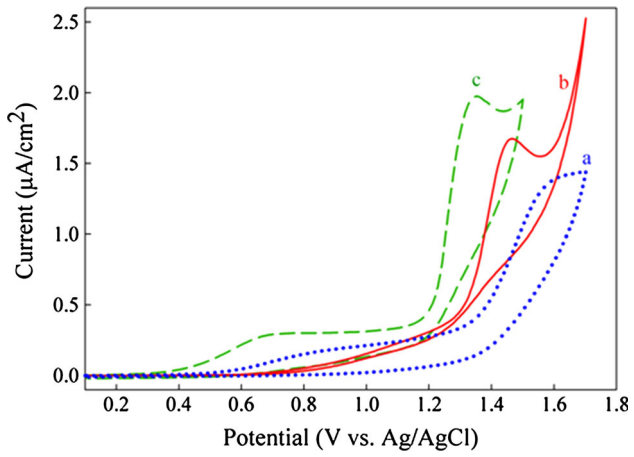

Fig. 2 Cyclic voltammograms of a mixture containing 0.5 mM CSSC and 2.0 mM CSH in PBS (pH 6.0) at **a** GCE, **b** CPE and **c** CILE, Scan rate 50 mV s^{-1}

The electrochemical oxidations of CSH and CSSC were compared by cyclic voltammetry at GCE, CPE and CILE in PBS (pH 6.0) (Fig. 2). CPE did not show any detectable oxidation signal for CSH and in the case of CSSC, a 100-mV positive shift was observed compared to CILE. A broad peak due to CSH oxidation on GCE appeared at a potential of 0.80 V which is 180 mV more positive compared to the observed CSH peak at CILE. CSSC oxidation at GCE showed 200 mV positive shift compared to the oxidation signal at CILE. In fact, CILE represented two well-defined peak-shaped oxidation signals at about 0.62 and 1.3 V with lower overpotential and higher current density compared to both GCE and CPE.

Figure 3 shows anti-fouling behavior of CILE by repetitive potential scans in a solution containing 1.0 mM of CSH and CSSC, respectively. The peak currents of CSH and CSSC reduced after the first scan. As shown in Fig. 3a, b, by stirring the solution for few seconds the diffusion layer was renewed and the initial signals recovered. This reveals that no surface fouling was taken place in the presence of the oxidized products at the electrode surface.

Effect of scan rate

The effects of scan rate on cyclic voltammograms of 200 μM CSH and CSSC at CILE are presented in Fig. 4a, b, respectively. The peak currents were measured at different scan rates within the range of 5–400 mV s^{-1}. It is observed that for both CSH and CSSC, the peak currents are enhanced by increasing the potential scan rates. The results indicate that the peak currents have a linear relation with square root of scan rates with correlation coefficients of 0.9977 and 0.9973 for CSH and CSSC, respectively. These results indicate that the nature of these two processes is diffusion controlled.

Effect of pH

The effects of pH on oxidation peak currents and oxidation peak potentials of CSH and CSSC were evaluated in the pH range of 4.0–9.0 in PBS. The results are shown in Fig. 5. As the solution pH increased, the oxidation peak current of CSH is decreased, while the oxidation peak current of CSSC reached a maximum value around pH 7.0 (Fig. 5a, b). The potential difference between the oxidation peaks of CSH and CSSC increased with rising pH (Fig. 5c). Since the observed potential differences between the two oxidation peaks in the whole pH range were sufficient for simultaneous determination, the optimum pH value was

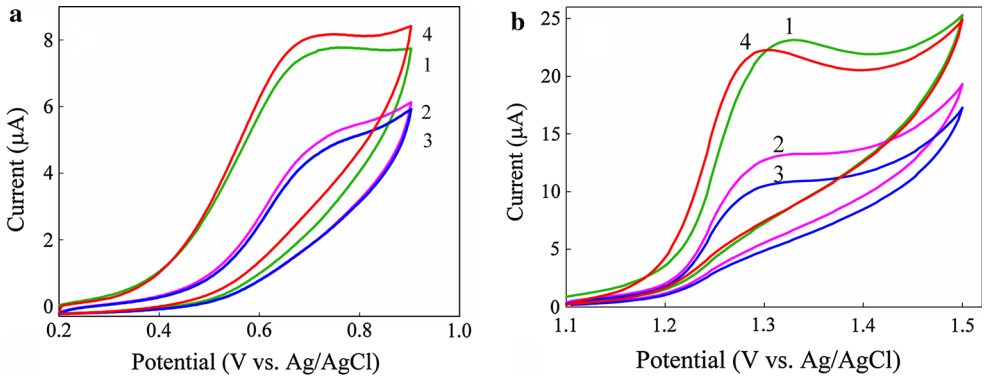

Fig. 3 Cyclic voltammograms (at CILE) of **a** 1.0 mM solution of CSH and **b** 1.0 mM solution of CSSC in PBS (pH 6.0) for repetitive consecutive scans (curves 1–3). Curve 4 after stirring the solution for a few seconds, scan rate 50 mV s^{-1}

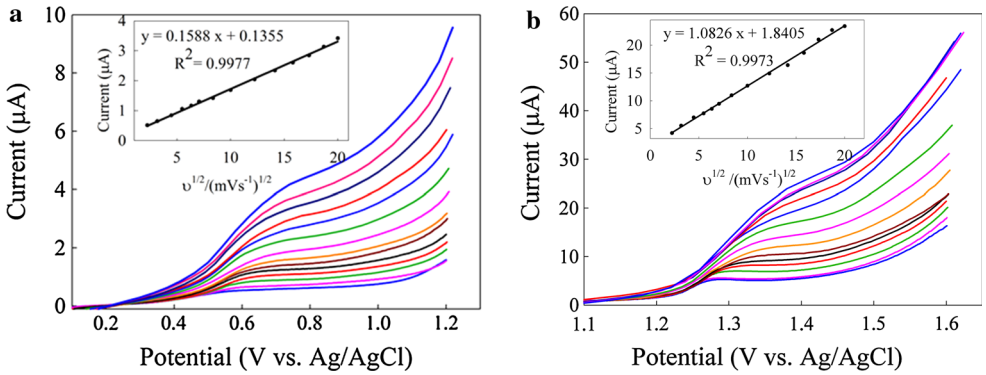

Fig. 4 Linear sweep voltammograms of CILE in **a** CSH (2.0×10^{-4} M) and **b** CSSC (2.0×10^{-4} M) solutions, at different scan rates: 5, 10, 20, 30, 40, 50, 70, 100, 150, 200, 250, 300, 350 and 400 mV s^{-1}, respectively. *Inset* plots of peak currents versus the square root of scan rates

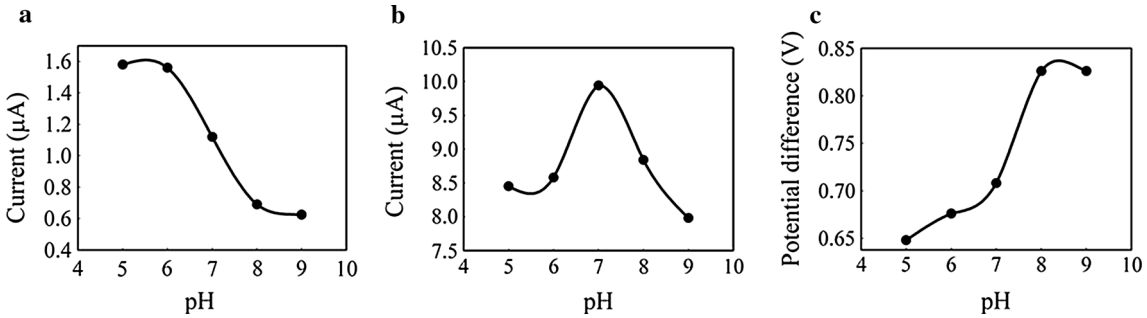

Fig. 5 Plot of the oxidation peak current of **a** CSH, **b** CSSC and **c** potential differences between CSH and CSSC oxidation peaks against pH in the solution containing 200 μM CSH and 100 μM CSSC at CILE. The peak potentials and peak currents were measured using linear sweep voltammetry recorded in a PBS solution

selected based on the peak currents of CSH and CSSC. Because the net current of CSH is lower than the corresponding current of CSSC in the whole pH range, therefore the net current of CSH was used for optimization of pH. As shown in Fig. 5a, pH 6.0 was selected as the optimum pH value. In this manner the detection limit of CSH electrooxidation could be decreased.

Analytical figures of merit

Linear sweep voltammetry was used in the potential range of 0.1–1.6 V for the simultaneous determination of CSH and CSSC. To show the linear relation between the net peak currents, as analytical signals, and the concentrations of CSH and CSSC, standard solutions of both species were prepared in PBS (pH 6.0). A good linear relationship was obtained between the oxidation peak currents and the concentrations of CSH and CSSC (Fig. 6). Calibration plots of the anodic peak currents versus concentrations of CSH and CSSC are presented in Fig. 6. For CSSC two linear ranges were obtained; 5.0–250 μM and 250–700 μM with correlation coefficients of 0.9976 and 0.9954, respectively (Fig. 6a). For oxidation peaks of CSH at 0.62 and 1.3 V the linear ranges were 1.0–450 and 10–450 μM with correlation coefficients of 0.9991 and 0.9975, respectively (Fig. 6b). The theoretical detection limits were

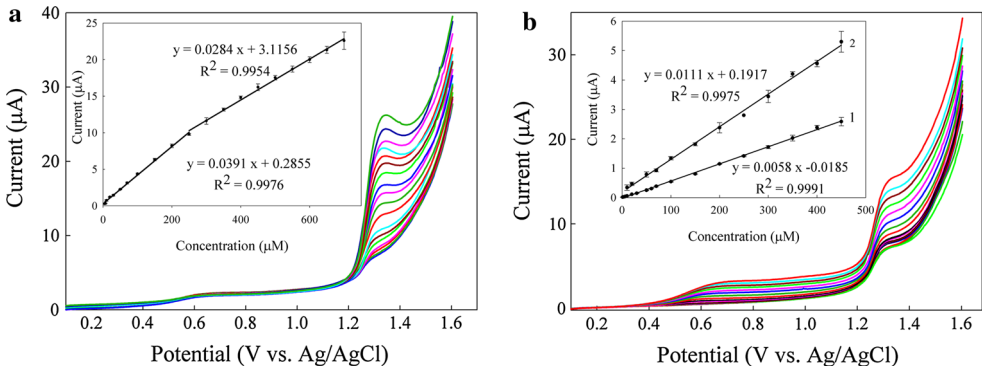

Fig. 6 Linear sweep voltammograms of **a** CSSC at CILE in the presence of 200 μM CSH in PBS (pH 6.0) (CSSC concentration: 5.0–250 μM). *Inset* calibration curves for CSSC at low and high concentration ranges. **b** Linear sweep voltammograms of CSH in the presence of 100 μM CSSC in PBS (pH 6.0). CSH concentration 1.0–450 μM. *Inset* calibration curves for oxidation peaks of CSH at (*1*) 0.62 V and (*2*) 1.3 V, respectively. The error bars indicate mean ± SD ($n = 5$)

obtained as 0.298 and 4.258 μM for CSH and CSSC, respectively, at a S/N ratio of 3.

The reproducibility was assessed by analyzing a solution containing 200 μM of both CSH and CSSC with three different CILEs. Satisfactory RSD values of 6.23 and 6.43 % were obtained for CSH and CSSC, respectively. These values indicate good reproducibility of the electrode for simultaneous determination of these two compounds.

Interferences

To investigate the selectivity of the sensor, the responses of the proposed electrode were studied toward various amino acids and other biomolecules that commonly exist in the real samples. The electrochemical responses of CILE toward fixed concentrations of CSSC and CSH (100 μM) were recorded in the presence of variable concentrations of interferences under optimum conditions (PBS, pH 6.0 and scan rate 50.0 mV s^{-1}). It is deduced from Tables 1 and 2 that most potential interferences including amino acids are negligibly responsive to the present sensor at least over twofold of the analytes concentration. The ascorbic acid interference in determination of CSH and CSSC is presented in Fig. 7.

Real sample analysis

To evaluate the applicability of the electrode, two real samples, artificial urine and nutrient broth (NB), were tested. NB is the most common chemically defined medium for microorganism growth. Artificial urine was prepared by the previously reported method (Chutipongtanate and Thongboonkerd 2010). For the analysis of the real samples, 100 μl of each real sample was diluted to 10 ml with PBS (pH 6.0) and was analyzed without any pretreatment using a standard addition procedure.

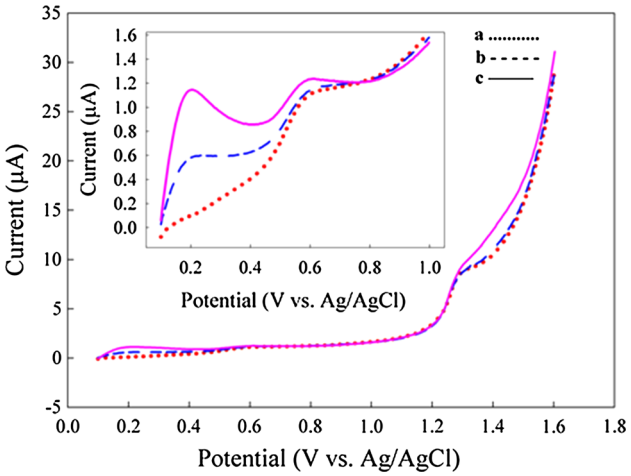

Fig. 7 Linear sweep voltammograms of a solution containing 100 μM CSH and CSSC in the **a** absence and **b** presence of 200 μM and **c** 500 μM ascorbic acid at CILE in PBS (pH = 6). *Inset* magnification of linear sweep voltammograms in potential of 0.1–1.0 V (vs. Ag/AgCl)

Table 1 Results of interference study for determination of 100 μM CSH

Species	Maximum tolerable concentration (μM)
Alanine, glycine, urea	50×10^3
Glutamic acid, lactic acid, creatinine	10×10^3
Arginine, lucin, valine, citric acid, tartaric acid	5×10^3
Phenyl alanine, histidine	1×10^3
Aspartic acid, tyrosine, methionine, uric acid, cystamine dihydrochloride	500
Oxalic acid	200
Glutathione, ascorbic acid, tryptophan	100

As shown in Tables 3 and 4, the results demonstrate satisfactory recoveries for determination of CSH and CSSC in two real samples.

Table 2 Results of interference study for determination of 100 μM CSSC

Species	Maximum tolerable concentration (μM)
Urea, creatinine	50×10^3
Alanine, glycine, lucin, valine	5×10^3
Arginine, aspartic acid, lactic acid, tartaric acid	1×10^3
Glutamic acid, uric acid	500
Tyrosine, phenyl alanine, methionine, citric acid, oxalic acid, ascorbic acid	200
Cystamine dihydrochloride, histidine, glutathione, tryptophan	100

Table 3 Determination of CSSC and CSH in NB

Added	Added (μM)	Found (μM)	Recovery (%)
CSH	0.0	ND	0.0
	10.0	10.14 ± 0.98	103.13
	30.0	31.88 ± 1.10	107.60
	60.0	57.41 ± 0.63	95.34
CSSC	0.0	ND*	0.0
	10.0	9.90 ± 3.05	98.97
	30.0	28.97 ± 1.50	95.35
	60.0	62.47 ± 2.44	105.14

Composition of NB: 0.5 % Peptone, 0.3 % yeast extract, 1.5 % agar and 0.5 % NaCl

ND not detected

± S ($n = 3$)

Table 4 Determination of CSSC and CSH in artificial urine

Added	Added (μM)	Found (μM)	Recovery (%)
CSH	0.0	ND	0.0
	10.0	9.89 ± 0.87	97.02
	30.0	30.34 ± 3.24	101.19
	60.0	60.08 ± 1.01	100.08
CSSC	0.0	ND*	0.0
	10.0	10.16 ± 4.21	101.60
	30.0	29.23 ± 0.89	95.33
	60.0	59.83 ± 1.50	99.34

ND not detected

± S ($n = 3$)

Conclusion

In the present work, unmodified CILE was used for direct simultaneous electrochemical determination of CSH and CSSC. This sensor exhibited attractive features such as ease of preparation, low cost, large potential window, renewable surface and resistance towards electrode fouling. Two well-defined oxidation peaks were observed for CSH and CSSC with significant potential difference. CILE shows wide linear ranges, high sensitivities and good reproducibility and repeatability towards CSH and CSSC oxidation. In addition, satisfactory results were observed for simultaneous determination of CSH and CSSC in real samples.

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Conflict of interest The authors declare that they have no conflict of interest.

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