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Enzymatic Determination of Glutathione Using Electrochemical Sensor Based on Cobalt Phthalocyanine Screen-printed Electrode

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Summary

Glutathione (GSH) has been detected with an electrochemical sensor. Different types of modified electrode material (screen-printed graphite, graphite-epoxy resin) have been compared. The best results for analytical determination were obtained with screen-printed graphite electrode modified with cobalt phthalocyanine (CoPC) at a potential of 0.20 V vs. saturated calomel electrode (SCE).

The analytical determination of GSH is based on the oxidation by a t-butylhydroperoxide catalysed by the enzyme glutathione peroxidase (GSH-Px) in solution, according to:

$$2GSH + ROOH \rightarrow GSSG + H_2O + ROH$$

Calibration curves of GSH standard solutions linear up to 30 μM were obtained in a solution of phosphate buffer (0.05 M phosphate ions, pH = 7) and 0.005 M EDTA.

This new procedure seems to be highly reproducible and has the distinct advantage of fast response time, linearity up to 30 μM of glutathione and a detection limit of 4 μM.

Keywords: glutathione, electrochemical sensor, screen-printed graphite electrode, cobalt phthalocyanine, glutathione peroxidase

Introduction

Reduced glutathione (GSH), the major soluble intracellular thiol found in mammals, is:

- an important factor in maintaining the integrity of red blood cells and
- an important cofactor in catabolism, metabolism and transport *(1,2)*.

A variety of electroanalytical methods have been developed for detection of GSH which is electroactive *(3-7)*. Methods based on mercury electrodes may be undesirable due to their toxicity or instability. Carbon alone requires undesirably large working potentials. But, carbon paste electrodes containing an electrocatalyst reduces the overpotential for the determination of GSH *(4,6-10)*.

We were interested in a device that would act as a sensor for reduced glutathione in biological materials *(1,2)*, in natural waters *(11)* and in grapes and musts *(12)*.

This analyte is a very important cofactor in many physiological processes and plays a key role in the detoxification of some common drugs *(1,2)*. Normal circulating concentration levels of GSH are 3.3 μM for plasma and 1.0 mM for whole blood. Changes in the concentration of GSH in biological tissues may be used as markers for certain disorders *(13)*.

Glutathione has been also detected in pore waters of marine sediments in samples from the estuary of the river Mersey in the North West of England which is sur-

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rounded by a large industrial area and then is heavily polluted with sewage. Preliminary analyses carried out revealed the presence of organic matter in the samples that interfered with the determination of GSH *(11)*.

Glutathione is also known to be an important grape component for its amount will define the biotechnology to be employed. It is possible to evaluate the origin of the grapes by the GSH level, indicating a site-related influence. The amounts of glutathione are to be affected by a number of technological operations, in particular, those which favor phenolic extraction *(12)*.

In the present study, electrochemical investigations with epoxy resin and screen-printed carbon electrodes modified or unmodified *(14)* has been carried out. Different types of electrocatalysts (Ru, Rh, Pt, Pd, CoPC) with the intention of optimising the conditions for the determination of GSH are used with the screen-printed electrodes. The evaluation of the modified screen-printed electrodes containing the most suitable electron mediator has also been done. The hydrodynamic voltammetry in stirred solution of GSH of epoxy resin and screen-printed carbon electrodes unmodified or modified with several catalysts are reported. This preliminary study was necessary to obtain information on stability and reproducibility of electrodes.

Screen-printed electrode doped with cobalt phthalocyanine (CoPC) proved to be a very suitable material and it was used as indicator for the reaction of *t*-butylhydroperoxide and GSH in the presence of glutathione peroxidase. This reaction was used as a base for detection of GSH in the concentration range up to 30 μM. The limit of detection was found to be 4 μM.

Experimental

Chemicals

CoPC and graphite were purchased from Fluka. GSH, glutathione peroxidase EC 1.11.1.9 (from bovine erythrocytes) were obtained from Sigma. Hydrogen peroxide (30%) and *t*-butylhydroperoxide (70%) were obtained from Carlo Erba and Prolabo, respectively.

The supporting electrolyte used for all the studies was potassium phosphate buffer (0.05 M phosphate ions, pH = 7) with 0.005 M EDTA. Solutions of GSH were prepared in this working buffer and protected from light during investigations. Higher pH values were not studied because GSH is particularly unstable in alkaline solutions *(15)* and it is easily oxidised by air *(4,16)*. However it is well known *(17,18)* that the reaction of GSH with glutathione peroxidase has a maximum rate at pH = 7. Therefore the buffer with this pH value was used throughout all the work.

The glutathione peroxidase lyophilized in vials containing 200 U (as defined by Sigma) was reconstituted with 1 mL of 0.05 M working buffer (pH = 7). This solution was then dialysed and stored in the dark at 4 °C.

Apparatus

Hydrodynamic voltammetry and amperometry were performed with the potentiostat AMEL model 559 HPLC detector with a recorder LINSEIS model L6512. Electrochemical detection was carried out using a three electrode cell incorporating a modified screen-printed electrode or epoxy resin electrode as the working electrode, a saturated calomel reference electrode and a platinum wire as counter electrode.

Amperometry in stirred solutions was used to investigate the hydrodynamic behaviour of the electrodes and to obtain calibration curves. The stirring of the solutions has scarce effect on the oxidation current of sulphydryl compounds, but is useful to mix the reagents. On increasing the stirring (from 1 to 6 arbitrary units), 8% increase in the current for GSH oxidation was observed.

Electrodes were printed with a model 245 screen-printer obtained from DEK (Weimouth, England) using different inks obtained from Acheson Italiana (Milano, Italy).

A graphite based ink (Electrodag 421), a silver ink (Electrodag 477 SS RFU) and an insulating ink (Electrodag 6018 SS) were used. The substrate was a polyester flexible film (Autostat HT5) obtained from Autotype Italia (Milano, Italy).

Electrodes construction

Epoxy resin electrodes. The epoxy resin composite electrodes were prepared by mixing the required amount of carbon black and epoxy resin. This was packed into glass tubes (5 mm). Electrical contact was made by pushing a wire down the tube and into the black of the mixture. Modified electrodes were prepared by adding 5% of CoPC. Before utilization, the electrodes were allowed to cure for 24 hours at room temperature and then the hardened surface was polished with a fine emery paper and washed with de-ionized water *(6,19,10)*.

Screen-printed electrodes. Each screen-printed electrode had a circular (5 mm in diameter) working area with 6 mm connecting strip. The inks were deposited on a substrate in a film of controlled pattern and thickness. A first layer of silver ink was printed on a polyester flexible film for the conductive pad. Over a part of the silver track, a carbon pad was positioned. Finally, an insulating layer with openings was printed, allowing electrical contact with the circuit at one end and between the carbon pad and the analyte solution at the other end. For the working surface, the inks were prepared using a graphite based ink manually mixed with 5% of the required mediator (Ru, Rh, Pd, Pt, CoPC). After every printing step, electrodes were cured at 110 °C for 10 minutes *(20)*.

Voltammetric procedure

Hydrodynamic voltammograms were recorded using the CoPC-modified screen-printed electrode in phosphate buffer solutions containing GSH in order to determine the electrocatalytic peak. They were obtained for modified and unmodified electrodes by amperometry in stirred solutions of 0.05 M phosphate buffer-EDTA (5 mL) without and with GSH. The applied potentials of the working electrodes were manually increased and the resulting steady-state current responses were measured for each plateau and plotted against the applied potential.

Results and Discussion

Voltammetric behaviour of GSH at modified or unmodified electrodes

The voltammetric evaluation of the different mediators for the electrocatalytic determination of GSH at the modified electrodes is described below.

Hydrodynamic voltammograms (Figs. 1-3) were recorded using modified and unmodified carbon-epoxy resin and screen-printed electrodes for solutions containing 97 μM of GSH. The potential of the working electrode was increased in 50 mV steps and the resulting currents were measured for each plateau and plotted against the potential value.

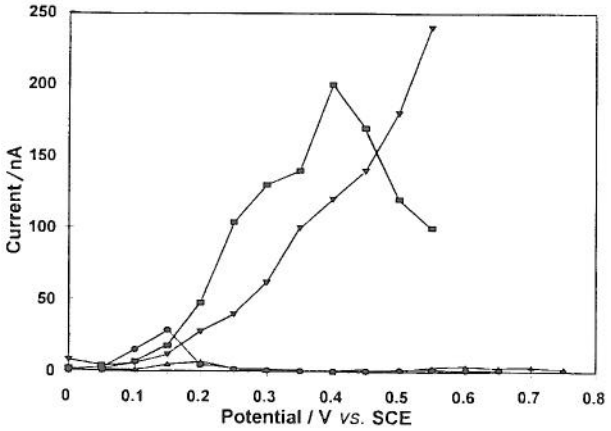

Fig. 1. Hydrodynamic voltammograms of GSH (97 μM) in phosphate buffer at pH=7 obtained using different electrodes: (▼) epoxy resin electrode, (■) epoxy resin electrode modified with CoPC, (▲) screen-printed electrode and (●) screen-printed electrode modified with CoPC.

The response for the screen-printed electrodes reveals a broad peak at approximately 0.20 V. This peak is seen only in the presence of GSH. The current measured is of greater significance with the modified screen-printed electrodes.

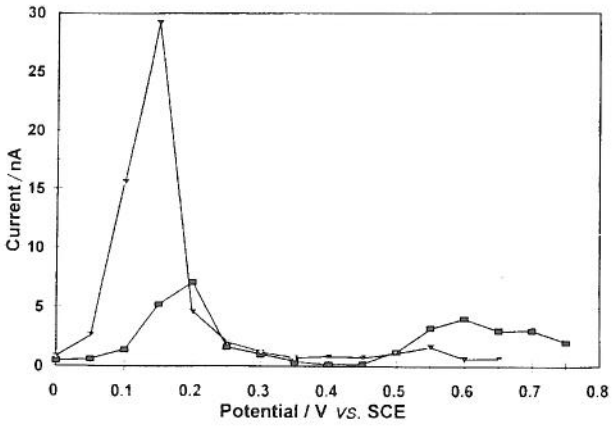

Fig. 2. Hydrodynamic voltammograms of GSH (97 μM) in phosphate buffer at pH=7 obtained using (▼) the modified (CoPC) or (■) unmodified screen-printed electrodes.

The results from both of the voltammetric studies indicate clearly that the potential necessary for the electrochemical detection of GSH can be decreased by using suitable electron mediators.

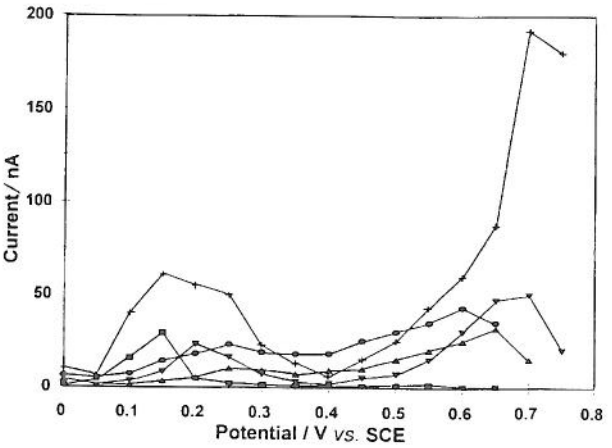

Fig. 3. Hydrodynamic voltammograms of GSH (97 μM) in phosphate buffer at pH=7 obtained using screen-printed electrodes modified with different mediators: (+) Ru, (▼) Rh, (●) Pt, (▲) Pd and (■) CoPC.

From Fig. 3 it can be seen that the highest sensitivity for glutathione determination was obtained with the electron mediator Ru (the current of 60 nA for a potential value of 0.15 V *vs.* SCE, with CoPC the current obtained was 30 nA for a potential of 0.15 V *vs.* SCE, while with Rh and Pt the current obtained was 20 nA for a potential of 0.20 V *vs.* SCE.

Table I. Coefficient of variation (CV) of modified screen-printed electrodes for glutathione concentration of 40 μM

Electron mediator used	CV/%
CoPC	3
Pt	8
Pd	9
Rh	9
Ru	12
–	13

However the screen-printed electrode using CoPC-modified graphite is characterized by the lowest coefficient of variation (CV) in the anodic current at potential 0.20 V *vs.* SCE (Table I), therefore this was chosen as promising mediator for the determination of GSH at pH = 7.

Hydrodynamic voltammograms (Fig. 4) were also recorded using the CoPC-modified screen-printed electrodes in solutions containing:

- GSH oxidized by air,
- GSH oxidized by air and hydrogen peroxide and
- GSSG.

The voltammograms indicate a large anodic response for hydrogen peroxide at 600 mV. With glutathione oxidized by air, the shoulder normally observed at 0.20 V is less significant. The GSSG did not show any

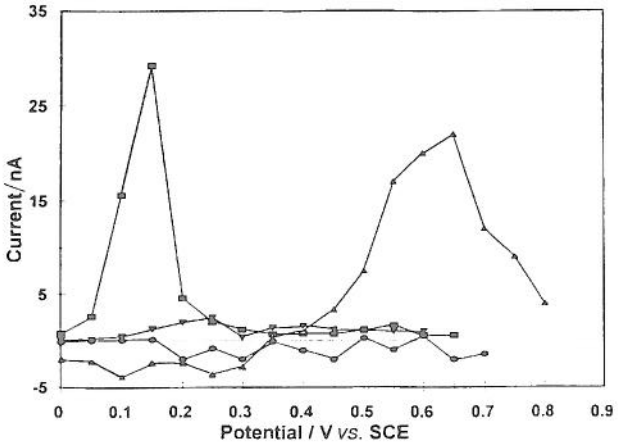

Fig. 4. Hydrodynamic voltammograms of (■) GSH (97 μM), (▼) GSH oxidized by air, (▲) GSH oxidized by air and hydrogen peroxide and (●) GSSG in phosphate buffer at pH=7 obtained using screen-printed electrode modified with CoPC.

current response over the potential range of interest. These experiments showed that the broad peak at 0.20 V corresponds to the oxidation of GSH.

Calibration

Calibration curves (*I vs.* concentration) were recorded using the method described previously, for the most promising modified electrodes. The concentrations studied covered the range 0.124 fM–5.5 nM GSH and a potential value of 0.20 V *vs.* SCE was applied. The calibration curves were obtained by adding volumes of a stock standard solution of GSH to the supporting electrolyte in the voltammetric cell.

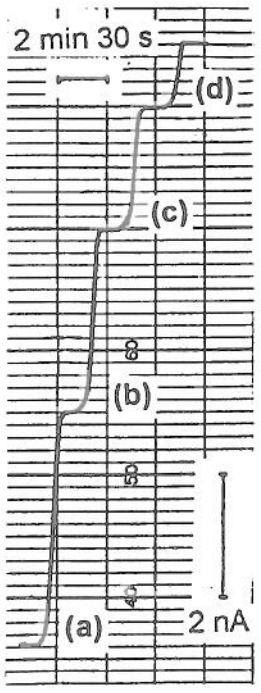

Fig. 5. Amperometric current response for injection of: (a) 136 μM, (b) 102 μM, (c) 68 μM and (d) 34 μM of GSH into the bulk supporting electrolyte (5 mL). E = 0.20 V *vs.* SCE.

Several additions of small volumes of stock GSH solution into the supporting electrolyte were performed with subsequent measurements of the current variation after each step. Fig. 5 illustrates the rapid response time (<60 s). The limit of detection was evaluated to be 0.05 μM of GSH.

Fig. 6 reports the calibration curves of the screen-printed electrode modified with CoPC and unmodified. The first has a linear part over 100 μM (screen-printed carbon-modified with CoPC), the second over 350 μM with different sensitivity (screen-printed unmodified).

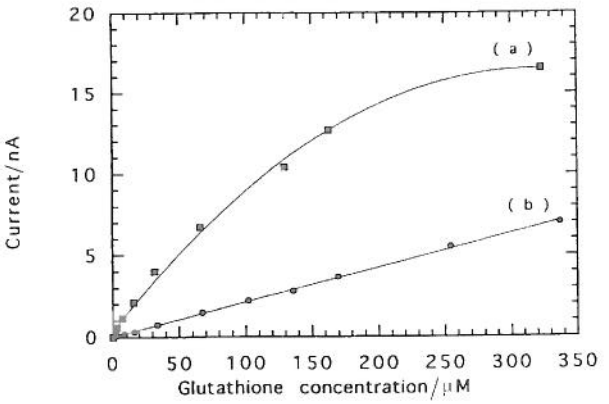

Fig. 6. Calibration graph obtained using amperometry for GSH at screen-printed electrode (a) doped with 5% CoPC and (b) unmodified electrode. E = 0.20 V *vs.* SCE.

The stability of the modified screen-printed electrode was evaluated by monitoring the current increase, due to addition of GSH to the supporting electrolyte to obtain a final concentration of 14 μM. This procedure repeated regularly over 12 days did not reveal significant loss of activity. The coefficient of variation for the current increase over this period was 2.9% (n = 60).

Voltammetric behaviour of hydroperoxides at the CoPC-modified screen-printed electrode

Unfortunately, such calibration curve cannot be applied for analytical determination of GSH in real samples. Many electroactive substances present in such samples can interfere. Therefore, we studied the selective enzymatic oxidation of GSH using hydrogen peroxide or *t*-butylhydroperoxide in the presence of glutathione peroxidase. Fig. 7 illustrates the amount of GSH consumed with increasing concentrations of peroxides without the enzyme.

In the absence of enzyme, significant direct oxidation of GSH was observed with hydrogen peroxide while the addition of *t*-butylhydroperoxide did not decrease the GSH concentration. Therefore *t*-butylhydroperoxide was the oxidant of choice.

Optimization of solution conditions for the enzymatic oxidation of GSH. Effect of temperature on the enzyme catalyzed reaction

The enzymatic oxidation using organic hydroperoxides can be represented by the following equations:

$$2\ GSH + ROOH \rightarrow ROH + H_2O + GSSG$$

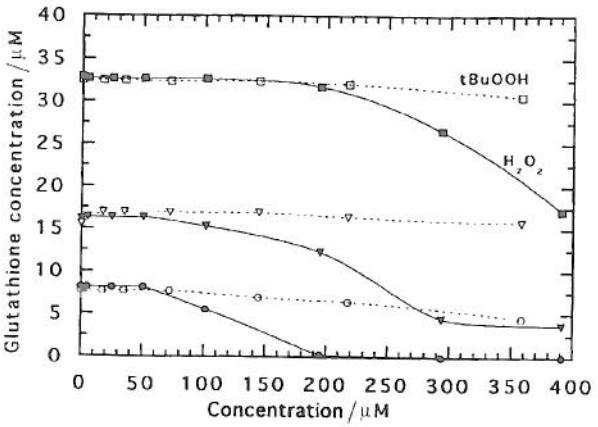

Fig. 7. Direct oxidation of GSH in presence of: hydrogen peroxide (—) and *t*-butylhydroperoxide (...) solutions. Initial GSH concentrations are: (□ and ■) 32 µM, (▽ and ▼) 16 µM and (○ and ●) 8 µM. E = 0.20 V *vs.* SCE.

This reaction is catalysed by glutathione peroxidase enzyme.

A typical anodic current response profile for the amperometric assay is shown in Fig. 8. There was an initial current step corresponding to the addition of GSH in buffer (0.05 M phosphate, pH = 7), which, after a short delay (solution mixed), achieved a steady-state response. At a chosen point, the enzyme and then the *t*-butylhydroperoxide were added and the decrease in current corresponding to the oxidation of GSH was monitored over several seconds.

Initial investigations were performed to determine if different temperatures (20 °C and 37 °C) could influence the current response for *t*-butylhydroperoxide standards. The concentration range studied was always selected to

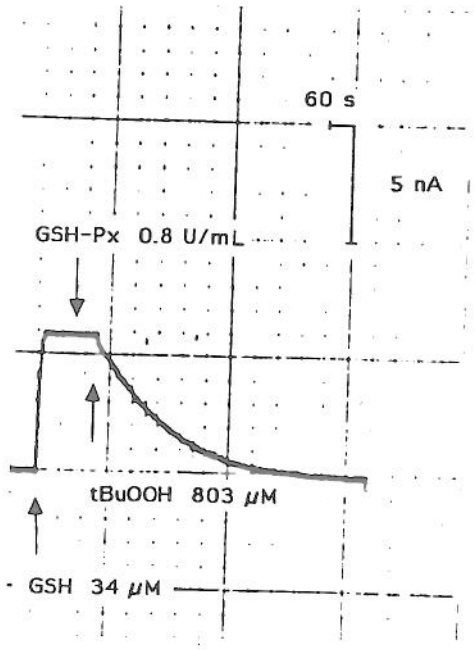

Fig. 8. Amperometric assay profile for the determination of GSH using glutathione peroxidase in conjunction with the CoPC-modified screen-printed electrode. E = 0.20 V *vs.* SCE.

cover physiological levels of GSH. These results revealed that the reaction rate does not increase with temperature.

The condition chosen for all future work to allow the enzymatic oxidation of GSH was to use 0.05 M phosphate buffer, pH = 7, at 20 °C with *t*-butylhydroperoxide.

Calibration of the enzymatic assay for GSH using glutathione peroxidase and amperometric detection in stirred solutions

A calibration plot for glutathione was obtained (Fig. 9), with the CoPC-modified screen-printed electrode (0.20 V *vs.* SCE) to follow the enzymatic oxidation of GSH under the selected solution conditions (0.05 M phosphate buffer, pH = 7) at room temperature. The concentration range studied (up to 30 µM) reflected the GSH levels that would be present in physiological solutions.

The limit of detection for the amperometric determination of GSH standard solution is 4 µM.

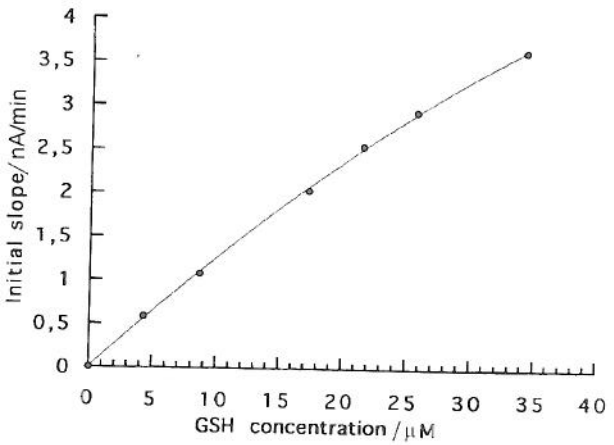

Fig. 9. Calibration plot of initial rate of current change (measured over the first 60 seconds) *vs.* starting GSH concentration. E = 0.20 V *vs.* SCE.

Conclusion

Wring *et al. (8)*, using CoPC modified carbon electrodes coupled with the glutathione peroxidase, reported a calibration plot over the concentration range 10 to 50 µM with a coefficient of variation of 7.28% (n = 5) for solutions containing 40 µM GSH.

Amperometric glutathione sensors based on H_2O_2 or Clark-type electrodes coupled with the glutathione oxidase gave calibration curves linear in the range 5 µM to 1 mM for the H_2O_2-based probe and 10 µM to 0.2 mM for the oxygen probe, and a relative standard deviations of 4.6% and 4.1%, respectively *(21)*.

Using a calorimetric flow injection with the glutathione sulfhydryl oxidase, the GSH calibration graph was linear from 0.5 to 10 mM and the relative standard deviation for 5.0 mM was 1.2% (n=5) *(22)*.

The voltammetric data and calibration results reported in this paper suggest that the screen-printed

graphite electrodes modified with the mediator CoPC are the most promising for determination of the reduced glutathione and for the study of the oxidation process for GSH.

The developed screen-printed graphite electrodes chemically modified with CoPC are both sensitive and reproducible. They have been successfully used for several hours for the determination of reduced glutathione without any significant deterioration in the measured current. The calibration curves obtained with standard GSH solutions are linear down to 100 μM with a detection limit of 80 nM. However this direct technique cannot be exploited with real samples because several electroactive compounds often are present and can interfere. Therefore we suggest to exploit the selectivity of the glutathione peroxidase which in presence of peroxide oxidizes only glutathione present in the samples.

The initial slope of the current is in this case the analytical signal. Linear calibration curves can be obtained in the concentration range of 1 to 40 μM with a detection limit of 4 μM. Such range fits the content of the most biological samples.

The described amperometric assay has demonstrated that fast analysis and great sensitivity can be achieved. The screen-printed CoPC electrodes are stable, economical, and when used in conjunction with the appropriate peroxidase, allow the monitoring of peroxidase-based enzymatic assays. The coefficient of variation for the enzyme-linked amperometric assay was 2.9% (n=6) for solutions containing 14 μM GSH. It appears promising that this technique could be used to produce a basis of selective sensors for the determination of GSH in food products (citrus fruit, grape, potato, etc.) and biological matrices (blood, plasma, bile, urine, etc.) in the future.

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Enzimsko određivanje glutationa s pomoću elektrokemijskog senzora temeljenog na tiskanoj elektrodi modificiranoj kobaltovim ftalocijaninom

Sažetak

Elektrokemijskim senzorom određen je glutation (GSH). Uspoređeni su različiti tipovi modificiranih materijala za elektrode (tiskani slojeviti grafit, grafit/ epoksi-smole). Najbolji su rezultati postignuti tiskanim elektrodama modificiranim kobaltovim ftalocijaninom (CoPC), pri potencijalu od 0,20 V prema zasićenoj kalomelovoj elektrodi (ZKE).

Analitičko određivanje GSH zasniva se na oksidaciji s t-butilhidroperoksidom katalizirano enzimom glutation-peroksidazom (GSH-Px) u otopini, prema jednadžbi:

$$2GSH + ROOH \rightarrow GSSG + H_2O + ROH$$

Kalibracijske krivulje za standardnu otopinu GSH linearne su do 30 μM, a dobivene su u otopini fosfatnog pufera (0,05 M fosfatni ion, pri pH = 7) i 0,005 M EDTA.

Taj je novi postupak reproducibilan, a bitna mu je prednost u brzom vremenu odziva, linearnosti do 30 μM glutationa i donjoj granici određivanja od 4 μM.