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Sulfite determination using sulfite oxidase biosensor based glassy carbon electrode coated with thin mercury film

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Abstract

The preparation of sulfite biosensor by immobilization of sulfite oxidase on a glassy carbon electrode coated with mercury thin film is described. Dissolved oxygen can be readily reduced at the glassy carbon electrode coated with mercury thin film. The working principle of the biosensor was based on monitoring decrease in the peak current due to the depletion in dissolved oxygen concentration according to the reaction of sulfite oxidase.

The biosensor allowed a low working potential of −0.24 V vs. Ag/AgCl. Immobilization parameters such as sulfite oxidase activity, gelatin amount, and glutaraldehyde percentage were investigated. The pH optimum of 7.0 was found when using phosphate buffer. Appropriate buffer concentration was found to be 0.05 M. Working temperature was accepted as 40 °C. The biosensor was stable at 40 °C for 3 h without loss of its initial activity. The calibration graph for the biosensor was linear between the concentration ranges of 2×10^{-4}–2.8×10^{-3} M sulfite. The results of sample analyses obtained with the biosensor agreed well with the enzymatic-spectrophotometric reference method.

Keywords: Sulfite oxidase; Sulfite; Food additives; Mercury thin film electrode; Biosensor

1. Introduction

Sulfite is widely used as an additive in food and beverages to prevent oxidation and bacterial growth and to control enzymatic reactions during production and storage. The abnormal levels of sulfite concentration is the symptoms of mild to severe harmful effects to skin, respiratory, or gastrointestinal signs and symptoms (Situmorang, Hibbert, Gooding, & Barnett, 1999; Vally, Carr, El-Saleh, & Thompson, 1999; Vally & Thompson, 2001).

Nowadays, due to the reported harmful effects towards hypersensitive people, sulfite content in food and beverages has been strictly limited in many countries. FDA has required warning labels on any food containing more than 10 mg/kg or beverage containing more than 10 mg/L of sulfite (Federal Register, 1986a, 1986b). Therefore, its determination in food is important and sensitive and selective methods are required for its determination.

Several methods have been reported for sulfite determination. Conventional method for sulfite determination includes the conversion of sulfite into sulphurdioxide and then to sulphuric acid which can be titrated with standard NaOH solution (AOAC Official Method 962.16, 16th Ed., 1995.) However, this method is time consuming and not selective and therefore more sensitive and selective methods are required.

Ion chromatography with electrochemical detection was used in conjunction with acid distillation for sulfite determination in food (Anderson, Warner, Daniels, & Padgett, 1986). High performance ion chromatography (HPIC) with conductivity detection by converting the sulfite content of

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food samples into sulphate (Ruiz, Santillana, De Alba, Nieto, & Garcia-Castellano, 1994). In a similar way, a capillary electrophoresis (CE) method was described for sulfite determination in food and beverages (Trenerry, 1996).

Another official method for sulfite determination was based on ion exclusion chromatography with direct current (dc) amperometric detection (AOAC Official Method 990.31, 16th Ed., 1995). This method was selective and samples need only be homogenized in buffer, filtered and injected for analysis. However, one drawback of the method was that fouling of the platinum working electrode occur which lead a significant decrease in the detector signal. This method was modified by using pulsed amperometric detection and more stable detector response was obtained (Federal Register, 1986a). Detection limit was 40 mg/L and RSD was less than 3% injection to injection.

Continuous methods have been applied for both sulphurdioxide and sulfite determination utilizing different manifolds and detection systems (Luq de Castro & Fernandez-Romero, 1995). A continuous-flow biosensor arrangement based on the dual immobilization of a biocatalyst on a controlled-pore glass and the reaction product on a resin support both packed in a flow-cell of a photometric detector was utilized for sulfite determination. The method was based on the enzymatic oxidation of sulfite in the presence of sulfite oxidase (SO) according to the following reaction:

$$SO_3^{2-} + O_2 + H_2O \rightarrow SO_4^{2-} + H_2O_2$$

The reaction product, hydrogen peroxide, was then converted by another enzyme to a cationic derivative which could be retained on an ion exchanger. The limit of detection was found to be 3 ng/mL and the method was applied to environmental samples.

Alternatively, the dissolved oxygen consumed in enzymatic reaction can be determined at mercury electrodes. Mercury thin film electrodes (MTFE) behave like a true mercury electrode and can be prepared easily. Besides, it provides a flat surface suitable for immobilization of enzyme. Previous study in this lab revealed that hydrogen peroxide could be determined in micromolar ranges within a response time of 3 min. Catalase was immobilized with gelatin by means of glutaraldehyde on the MTFE surface and the biosensor response was monitored by following the reduction peak of dissolved oxygen at −0.24 V (Ertaş, Timur, Akyılmaz, & Dinçkaya, 2000).

Present study describes a method for specific determination of sulfite in food samples by a sulfite oxidase biosensor based on MTFE following decrease in the peak current due to the depletion in dissolved oxygen concentration according to the reaction above.

2. Materials and methods

2.1. Apparatus and reagents

The chemicals used were of analytical reagent grade. Voltammetric analysis was carried out with a Metrohm 694 VA Processor and Stand. A three-electrode system was used including Ag/AgCl reference electrode and platinum auxiliary electrode. The working electrode was a mercury thin film electrode deposited on a glassy carbon (GCE) support with a 3 mm diameter supplied from Metrohm. Sonication was made with Ultrasonic LC 30.

2.2. Procedure

The GCE surface was polished on a piece of velvet with alumina slurry, rinsed with distilled water and then sonicated for 10 min. The electrodes were placed in a voltammetric cell containing 10 mL of distilled water and 10 mL of mercury plating solution (200 mg/L $HgCl_2$ in 2 M HCl). Nitrogen gas was bubbled through the cell for 5 min and then the electrode was conditioned by scanning the potential between −0.1 and 1.2 V for several times. Mercury deposition was maintained by applying a potential of −0.8 V for 90 s while the electrode was being stirred at 1800 rpm.

Sulfite oxidase enzyme (0.5 U) and gelatin (1 mg/50 μL) were mixed at 38 °C in 0.05 M phosphate buffer (pH 7.0) and 50 μL of this solution was spread over the GCE surface plated with MTF and allowed to dry at 4 °C for 1 h. Finally the electrode was immersed in 2.5% glutaraldehyde solution in 0.05 M phosphate buffer for 5 min.

The method developed was adopted for the determination sulfite in several food samples. Food samples analyzed were chosen as foods containing sulfite additives in the market. The sample preparation buffer was adopted from AOAC Method 990.31 (AOAC Official Method 990.31, 16th Ed., 1995). It was alkaline so that both free and bound sulfite can be extracted. Mannitol was included to slow the oxidation of sulfite to sulfate. An appropriate volume of mannitol buffer was added to sample. The mixture was blended at high speed for about 1 min. After homogenization, the sample was centrifuged for 15 min at 4500 rpm. The resulting supernatant was used for the analyses. Liquid samples were just diluted in the buffer prior to injection. The biosensor and reference methods were used on the same samples.

The values are given in Table 1 comparatively with a reference method including enzymatic and spectrophotometric detection.

3. Results and discussion

Initial studies were conducted to examine the reduction peak of dissolved oxygen on the MTFE. The electrode surface was first covered with gelatin alone and immersed in voltammetric cell containing pH 7.5 phosphate buffer solution. Then, the potential was scanned several times from 0 to −0.75 V. Dissolved oxygen content of the solution gave a well formed reduction peak at −0.24 V being at more negative potentials than that of bare MTFE. Oxygen reduction peak was observed at −0.24 V so that the interference effects of certain substances which could be reduced at high potentials could be avoided.

Table 1
Comparatively results of real sample analyses obtained with the present biosensor and the reference method (AOAC Official Method 962.16, 16th Ed., 1995)

Sample type	Biosensor method[a]	RSD (%)	Reference method[a]	RSD (%)	Recovery (%)	Relative error (%)
Sesame cracker	114.0 ± 7.5 mg/kg	6.6	120.0 ± 1.7 mg/kg	1.4	−4.75	−5
Cracker	126.5 ± 4.1 mg/kg	3.2	131.2 ± 2.0 mg/kg	1.5	−8.75	−3.6
Ready soup	190.0 ± 10.5 mg/kg	5.5	183.0 ± 7.0 mg/kg	3.8	−2.5	3.9
Vinegar	1525 ± 22 mg/L	1.4	1560 ± 13 mg/L	0.84	+6	−2.2

[a] Mean of three determination.

The peak current was measured subsequently and no significant change in peak heights was observed after 25 scan. Addition of sulfite in high concentrations (5×10^{-3} M) into the cell had no effect on the peak height discarding the interference of sulfite on the reduction peak of oxygen in the absence of enzyme. Measurement principle of the biosensor system was based on monitoring decrease in the peak current due to the depletion in dissolved oxygen concentration according to the reaction of sulfite oxidase. The rate of consumption of oxygen was proportional to the level of sulfite. The difference between the first (without sulfite) and the second (with sulfite) peak current values was used to quantify the change of oxygen peak current. Measurements were carried out by the change of oxygen peak current related to sulfite concentration added to the electrochemical reaction cell.

Then the biosensor studies were initiated and certain parameters related with the immobilization procedure and working conditions were investigated.

3.1. Optimization studies of bioactive membrane components of the biosensor

The effect of SO enzyme activity on the peak current in the presence of sulfite ion was examined while the gelatin and glutaraldehyde percentages were kept constant being 1 mg/50 μL and 2.5%, respectively. The response of the biosensor was taken as the peak current difference of dissolved oxygen in the absence and presence of sulfite (ΔI_p). Fig. 1 shows biosensor response obtained at differing enzyme activities. Similar results were obtained with 0.5 and 1.0 U/50 μL and 0.5 U/50 μL was chosen since it gave a wider range of linear response for sulfite concentration. Higher enzyme activity resulted in a gradual decrease in biosensor response probably due to the more intensive cross linking which constituted a diffusion barrier for the substrate.

Gelatin and glutaraldehyde amount was optimized as 1 mg/50 μL and 2.5%, respectively. Related graphs are given in Figs. 2 and 3. The percentages of glutaraldehyde were 1.25%, 2.5%, and 5%. These studies showed that optimum sulfite oxidase activity, optimum gelatin quantity, and optimum glutaraldehyde percentage were 0.5 U, 1 mg, and 2.5%, respectively. In all experiments, enzyme activity, gelatin quantity, and glutaraldehyde percentage were kept constant at 0.5 U, 1 mg, and 2.5%, respectively.

3.2. Optimization studies of working conditions

Working conditions were also optimized. First of all, optimum pH was searched by using phosphate buffer solutions in a pH range of 6.0–8.0. The best results were obtained at pH 7.0. In addition to phosphate buffer, Tris–HCl, and triethanolamine buffer systems at pH 7.0

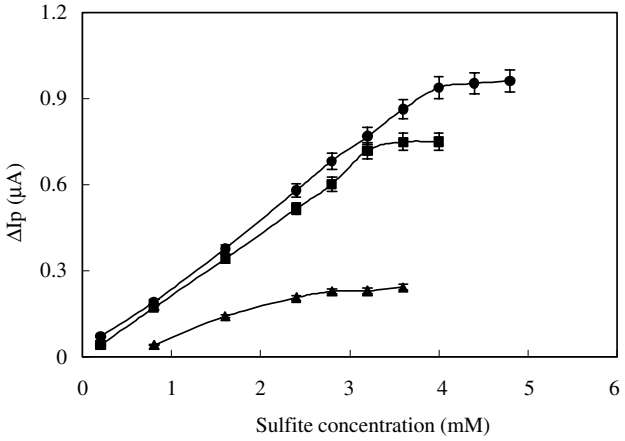

Fig. 1. The effect of sulfite oxidase activity on the biosensor response. [Sulfite oxidase activities; -●-●-: 0.5 U/50 μL, -■-■-: 1 U/50 μL, -▲-▲-: 0.25 U/50 μL. Gelatin amounts and glutaraldehyde percentages were kept constant as 1 mg and 2.5%, respectively. Working conditions: pH 7, 0.05 M phosphate buffer, 35 °C.]

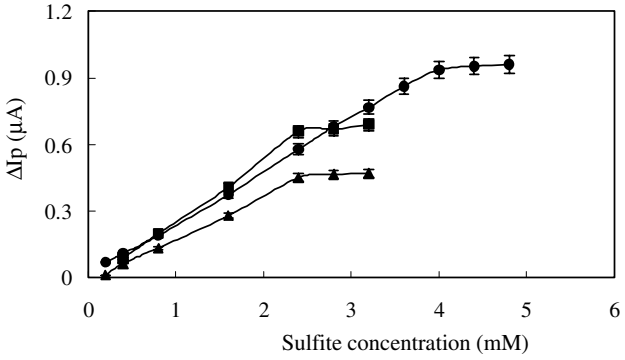

Fig. 2. The effect of gelatin amount on the biosensor. [Gelatin amounts; -●-●-: 1 mg/50 μL, -■-■-: 0.5 mg/μL, -▲-▲-: 2 mg/50 μL. Sulfite oxidase activities and glutaraldehyde percentages were kept constant as 0.5 U/50 μL and 2.5%, respectively. Working conditions: pH 7, 0.05 M phosphate buffer, 35 °C.]

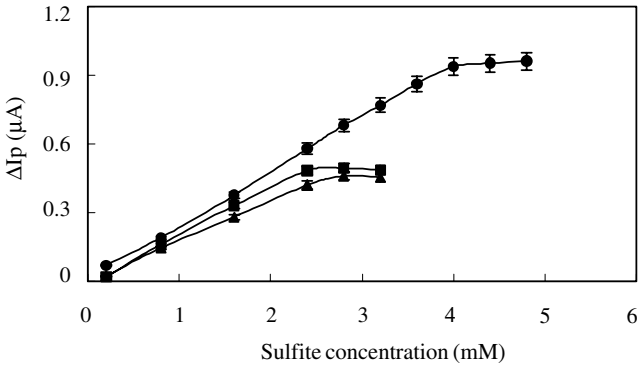

Fig. 3. The effect of glutaraldehyde percentage on the biosensor. [Glutaraldehyde percentages; -●-●-: 2.5%, -■-■-: 1.25%, -▲-▲-: 5%. Sulfite oxidase activities and gelatin amounts were kept constant as 0.5 U/50 μL and 1 mg/50 μL, respectively. Working conditions: pH 7, 0.05 M phosphate buffer, 35 °C.]

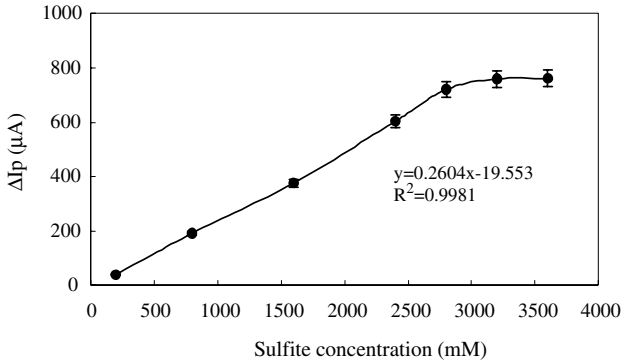

Fig. 4. Calibration graph for sulfite. [Sulfite oxidase activity, gelatin amount and glutaraldehyde percentage were constant as 0.5 U/50 μL, 1 mg/50 μL, and 2.5%, respectively. Working conditions: pH 7.0, 0.05 M phosphate buffer, 40 °C.]

were tested. Among these systems phosphate buffer was preferred in terms of sensitivity.

The concentration of buffer solution was another parameter that might affect the biosensor response and the percent activity change vs. buffer concentration was investigated. The phosphate buffer gave the maximum response at 0.05 M.

The temperature effect on the biosensor response was also examined. The biosensor response was increased with temperature up to 45 °C. On the other hand it is well known that enzyme activity shows a decline at high temperatures. Therefore 40 °C was chosen as the working temperature. Thermal stability of the biosensor was tested by incubating biosensors prepared by the same procedure and keeping one at 35 °C and the other at 40 °C. No significant difference between the responses of both biosensors was noted after 3 h. Lastly, storage stability studies revealed that the biosensor response was found stable over 10 days after it was prepared and 17% activity loss was recorded after 13th day.

3.3. Analytical characteristics

Under the optimal conditions given above, the calibration graph for sulfite was constructed (Fig. 4). The graph was linear between the concentration ranges of 2×10^{-4}–2.8×10^{-3} M.

Repeatability of the biosensor was tested upon 10 repetitive measurements for 1.6×10^{-3} M sulfite and RSD was calculated as 4.1%. This loss might be arisen both from the activity loss of the enzyme and deterioration of mercury film as well.

Interference study included the effect of bisulphite, metabisulphide, and sulphate ions on the biosensor response. No signal was obtained for sulphate. Other two ions are converted into sulfite ion in aqueous solutions and therefore they can be detected as sulfite. Therefore, selectivity was maintained for solid samples which could be treated with aqueous solutions.

3.4. Real sample analysis

As follows from the Table 1, the results of the developed method were in good agreement with those of reference method. Relative errors between the results obtained with the biosensor and the reference method were within acceptable limits.

However, the results were higher than permitted levels specified as 50 mg/kg for crackers and 170 mg/L for vinegar. Sensitivity of the method developed was adequate for determining sulfite in food samples well above the permitted levels.

4. Conclusion

In conclusion, we have demonstrated a simple and effective biosensor to determine sulfite. The good analytical properties such as fast response, long-time stability, and a good detection range. The construction of the biosensor, measurements, and cost of a biosensor were very simple and low. These advantages proposed that the biosensor based on sulfite oxidase could be applied successfully for determination of sulfite in foods.

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