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A sensitive and selective on-line amperometric sulfite biosensor using sulfite oxidase immobilized on a magnetite-gold-folate nanocomposite modified carbon-paste electrode

Wongduan Sroysee, Kitayanan Ponlakhet, Sanoe Chairam, Purim Jarujamrus, Maliwan Amatatongchai *

Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ubon Ratchathani University, Ubon Ratchathani 34190, Thailand

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ABSTRACT

We describe a novel amperometric sulfite biosensor, comprising a carbon-paste electrode (Fe_3O_4@Au-Cys-FA/CPE) modified with immobilized sulfite oxidase (SOx) on a gold-coated magnetite nanoparticle core, encased within a conjugated folic acid (FA) cysteine (Cys) shell. The biosensor electrode was fabricated using a polydimethylsiloxane (PDMS) and mineral oil mixture as binder, which also enhances the physical stability and sensitivity of the electrode. The developed biosensor displays good electrocatalytic activity toward oxidation of H_2O_2, which occurs by an enzymatic reaction between SOx and sulfite. The Fe_3O_4@Au-Cys-FA electrode exhibits good electrocatalytic activity, and has good retention of chemisorbed SOx on the electrode because of its large surface area. Sulfite was quantified using amperometric measurements from the Fe_3O_4@Au-Cys-FA/CPE biosensor, and using an in-house assembled flow cell at +0.35 V (vs. Ag/AgCl), with a phosphate buffer carrier (0.10 M, pH 7.0) at a flow rate of 0.8 mL min^{-1}. The system detects sulfite over the range 0.1–200 mg L^{-1} (r^2=0.998), with a detection limit of 10 μg L^{-1} (3σ of blank). The system exhibits acceptable precision (%R.S.D.=3.1%), rapid sample throughput (109 samples h^{-1}), and good stability (2 w). The developed biosensor shows satisfactory tolerance to potential interferences, such as sugars, anions, ascorbic acid, and ethanol. We applied the developed method to the determination of sulfite content in wines and pickled food extracts, and our results are in good agreement with those obtained by the standard iodometric method.

1. Introduction

Sulfite ion (SO_3^{2-}) has wide application as a food preservative and as an antioxidant in food and beverages to inhibit enzymatic and non-enzymatic activities that cause browning, and to suppress the growth of microorganisms during storage [1–4]. However, sulfite is potentially toxic and may cause adverse reactions in sulfite-sensitive, asthmatic individuals [1–3]. The US Food and Drug Administration (FDA), recommends warning labels on any food that contains sulfite at concentrations greater than 10 mg kg^{-1}, and on beverage and wine products containing in excess of 10 mg L^{-1} sulfite [1–5]. To accurately control the quality of manufactured products, a simple, sensitive, and accurate analytical method for the determination of sulfite is required.

Various techniques for quantitative analysis of sulfite are available, including fluorescence spectroscopy [6], high-performance liquid chromatography in combination with ultraviolet spectrophotometry [7], and chemiluminescence spectroscopy [1,8]. However, these techniques are time-consuming, need sample pre-treatment and reagent preparation, and may suffer from lack of sensitivity or precision, and so, the development of more sensitive and selective methods is crucially important for routine monitoring. Electrochemical measurements, using enzymatic modification at metallic electrodes, carbon/graphite electrodes, or chemically modified electrodes, offer several benefits over traditional methods, including greater selectivity, sensitivity, and reliability, and offer the possibility of on-line applications [9]. Fabrication of ch7emically modified electrodes allows electrocatalysis of sulfite oxidation reactions and reduction of the sulfite oxidation potential [10–12], although the reported selectivity, sensitivity, and reliability are poor. Thus, it is of considerable interest to develop an enzyme-based biosensor for the detection of sulfite ion; this would provide high selectivity, sensitivity, and accuracy in the

* Corresponding author.
E-mail address: maliwan.a@ubu.ac.th (M. Amatatongchai).

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quantification of sulfite, without interference from sample constituents or fouling of the electrode. A high degree of selectivity toward sulfite is achievable by using sulfite oxidase (SOx) enzyme to convert sulfite to sulfate and H_2O_2 [9, 13–15]. There are several recent reports of amperometric sulfite-biosensors using SOx immobilized on various electrodes [13–15]. These biosensors use a SOx catalyzed reaction for sulfite detection; molybdenum active sites in SOx undergo both oxidation and reduction during oxidation of sulfite to sulfate [15]. Sulfite determination can be performed by detecting the SOx-electrode signal directly, or indirectly by monitoring the depletion of oxygen or formation of H_2O_2 [9,14,15]. The simplest approach might be to measure the generation of peroxide. However, the high operating potential of such a biosensor, can result in oxidation of other components within the sample matrix, which will generate nonspecific signals. Electron-transfer mediators such as tetrathiafulvalene (TTF) [15], cytochrome c (Cyt c) [13,14], and ferro/ferricyanide [16] provide greater selectivity for sulfite determination by H_2O_2 detection. Minimization of the biosensor operating potential is based on either regeneration of the mediator, or on catalysis of H_2O_2 formation, or both [9,15].

Magnetite (Fe_3O_4) nanoparticles have useful physical and chemical properties including being superparamagnetic, having a large surface area, strong adsorption characteristics, good mechanical stability and electrical conductivity, and superb electrocatalytic activity [17–19]. There are many approaches available for the functionalization or modification of Fe_3O_4 surfaces for biomedical applications. Methods include using metals, oxides, organic monolayers, and polymers [17]. One of the most promising modifications is to use gold-coated magnetite (Fe_3O_4@Au). This material is simple to prepare, offers the possibility of bioconjugation, and has good biocompatibility for drug delivery applications [17,18]. Samphao et al.[20] and Li et al. [21] reported the application of Fe_3O_4@Au to improve electrocatalytic activity in enzyme-based sensors for the analysis of glucose [20] and *Escherichia coli* [21]. Fe_3O_4@Au nanoparticles were integrated into composite materials to immobilize glucose oxidase [20] and horseradish peroxidase [21] via chemisorption or physisorption, and then used to modify screen printed electrodes [20] and glassy carbon electrodes [21]. Recently, Karamipour et al. [22] reported the synthesis of folic acid (FA)-cysteine (Cys)-conjugated gold-coated magnetite nanoparticles (Fe_3O_4@Au-Cys-FA) using L-cysteine as a bi-functional linker for attachment to the gold surface via its thiol group. The nanomaterial was characterized and designed its utilization to drug delivery applications. However, there are no reports of catalyst or biosensor applications using Fe_3O_4@Au-Cys-FA nanocomposite materials.

In this work, we report the synthesis of Fe_3O_4@Au-Cys-FA nanoparticles and fabrication of a sensitive and selective sulfite biosensor. The biosensor was constructed by immobilizing sulfite oxidase on a Fe_3O_4@Au-Cys-FA nanocomposite-modified carbon paste electrode via direct chemisorption. To our knowledge, this is the first report of using Fe_3O_4@Au-Cys-FA to enhance the electrocatalytic activity and enzyme immobilizing performance of a sulfite biosensor. The Fe_3O_4@Au-Cys-FA/CPE provides significant improvement in biosensor performance. The large surface area and unique nanostructure of Fe_3O_4@Au-Cys-FA provide good electrocatalytic activity and facilitate retention of SOx on the electrode, resulting in high sensitivity and selectivity by the biosensor. When applied to on-line amperometric detection of sulfite in a flow injection (FI) system, the sensor successfully quantified sulfite in pickled food extracts and wine samples. The biosensor exhibits excellent sensitivity, selectivity, and stability with a sample throughput of 109 samples per hour.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were of analytical reagent grade. Deionized-distilled water (Water Pro-PS, USA) was used for preparation of standards and reagents. Chicken liver sulfite oxidase (SOx, 30–70 U mg^{-1}) was purchased from ProNique Scientific, Inc. (Castle Rock, USA). Sodium sulfite (Na_2SO_3) and horse heart cytochrome c (Cyt c, 90%) were purchased from Sigma-Aldrich (St. Louis, USA). Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4 \cdot 3H_2O$), 99.99%), N, N′-dicyclohexylcarbodimide (DCC), folic acid ($C_{19}H_{19}N_7O_6$, 97% purity), L-Cysteine ($C_3H_7NO_2S$), tri-sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, ferric nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$), ferrous sulfate heptahydrate ($Fe(SO_4)_2 \cdot 7H_2O$), and graphite powder were purchased from Acros Organic (Geel, Belgium). Poly(dimethylsiloxane) (Sylgard® 184) were purchased from Dow Corning (Wiesbaden, Germany).

2.2. Apparatus

2.2.1. Cyclic voltammetry

Cyclic voltammetry (CV) was performed in batches using an eDAQ potentiostat (model EA161, Australia) equipped with e-corder (model 210), and e-Chem software v2.0.13. For CV measurements, we used a self-assembled three-electrode cell, comprising a Fe_3O_4@Au-Cys-FA-SOx carbon-paste working electrode, an Ag/AgCl (sat.) reference electrode, and a platinum wire counter electrode. The carbon paste electrode (CPE) active surface area was approximately 0.031 cm^2 (inner diameter 0.2 cm). Electrochemical measurements were performed in phosphate buffered solution (PBS) (0.1 M, pH 7).

For the cyclic voltammetry study, we assembled electrodes by packing Fe_3O_4@Au-Cys-FA-SOx paste inside a glass tube (inner diameter 0.2 cm, length 7.5 cm) and fixing a copper wire in place using epoxy resin. The Fe_3O_4@Au-Cys-FA electrode surface was polished to a smooth surface with weighing paper. The electrode was encapsulated by drop casting 0.01% Nafion® solution in water (10 μL) onto the polished electrode surface. Finally, the electrode was dried at room temperature, and then stored at 4 °C in a refrigerator until required for use.

2.2.2. On-line amperometric detection using a simple flow injection system

The flow injection system used for amperometric detection of sulfite [23] comprised a Shimadzu pump (model LC-10CE, Japan), a Rheodyne injector (model 7725, USA) fitted with a 20 μL sample loop, and an electrochemical detector (ECD). For the measurements, we used an eDAQ potentiostat (EA161), equipped with an e-corder 210, Chart software v5.5.11, and our in-house three-electrode flow cell. The Fe_3O_4@Au-Cys-FA carbon-paste biosensor served as the working electrode, Ag/AgCl as the reference electrode, and a stainless steel tube as the counter electrode. The electrode area in the flow cell was approximately 0.060 cm^2.

The Fe_3O_4@Au-Cys-FA/CPE electrodes were assembled by packing composite paste inside the working electrode block of the thin-layer flow cell (Fig. 1B). The surface of the electrode was covered with a 0.01% Nafion® film (40 μL), formed by drop casting and drying at room temperature. In the ECD, a silicone rubber gasket (flow channel = 0.1×0.6 cm^2) provided a spacer between the base of the cell and the working electrode. The analyte solution passes through an inlet passage in the base, and along a channel in the gasket contacting the biosensor, then to the outlet.

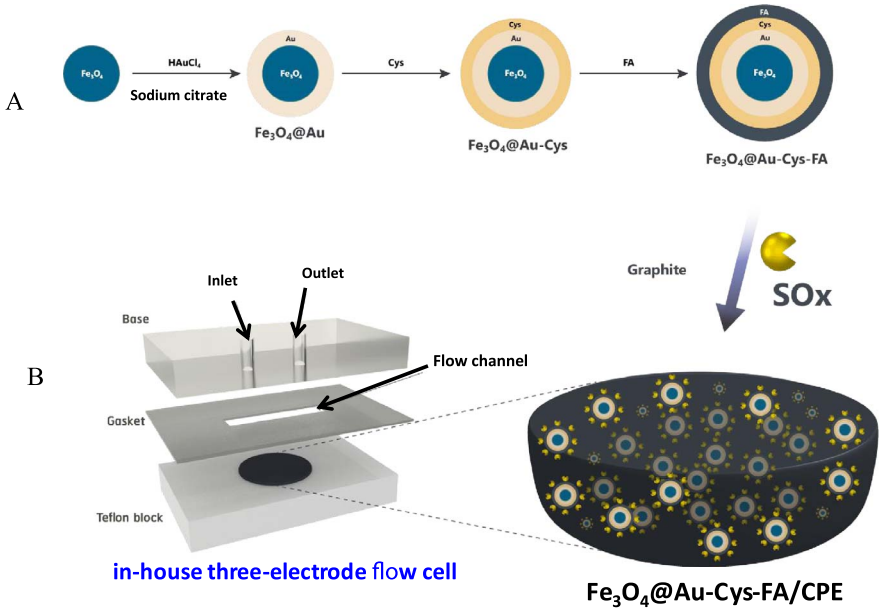

Fig. 1. Preparation of sulfite biosensors (A) Fe_3O_4@Au-Cys-FA nanocomposites with immobilized Sox, and (B) Fe_3O_4@Au-Cys-FA modified carbon-paste electrode.

2.2.3. Characterization

A JEM-1230 transmission electron microscope (TEM; JEOL, Japan) was used to observe the size and morphology of Fe_3O_4 and Fe_3O_4@Au nanoparticles. Elemental analysis data for Fe_3O_4 and Fe_3O_4@Au, and EDS spectra for Fe_3O_4@Au-Cys-FA nanoparticles were collected using energy dispersive X-ray spectroscopy (INCAx-sight, UK) coupled to a scanning electron microscope (SEM, JEOL, JSM-6460LV, Japan) operated under vacuum at an accelerating voltage of 20 kV.

2.3. Preparation of the sulfite biosensor

2.3.1. Preparation of magnetite nanoparticles (Fe_3O_4)

Magnetite nanoparticles were prepared by co-precipitation, following a method described by Karamipour et al. [22] and Kouassi et al. [24]. Briefly, $Fe(NO_3)_3 \cdot 9H_2O$ (0.02 mol) and $FeSO_4 \cdot 7H_2O$ (0.01 mol) were dissolved in HCl (60 mL, 0.2 M). The mixed solution of ferrous and ferric salts was then added dropwise into aqueous NaOH solution (100 mL, 1 M, purged with nitrogen gas) with vigorous stirring at 70 °C under nitrogen. Stirring was continued for a further 2 h. The formed black Fe_3O_4 precipitates were collected by an external magnetic field, washed with water three times by magnetic decantation, and then dried in a desiccator.

2.3.2. Preparation of Fe_3O_4@Au

Fe_3O_4@Au nanoparticles were prepared following literature methods [22,25], with some modifications. First, Fe_3O_4 nanoparticles (30 mg) were dispersed in 10 mL of water in an ultrasonic bath for 30 min Next, Au^{3+} was reduced to Au° on the surface of Fe_3O_4 using tri-sodium citrate. The dispersion was added into a 250 mL round-bottom flask containing 40 mL of distilled water, and 20 mL of 0.1% $HAuCl_4$ was added while stirring. The reaction was bought to a boil under reflux. Upon boiling, 4 mL of 1 wt% tri-sodium citrate was rapidly added to the stirred solution. The solution color changed from brown to reddish brown and the stirred mixture was kept at reflux for a further 15 min The red-brown Fe_3O_4@Au nanoparticles were separated using an external magnetic field, washed with water three times by magnetic decantation, and then dried in a desiccator.

2.3.3. Preparation of Fe_3O_4@Au-Cys-FA

Fig. 1A illustrates the preparation of Fe_3O_4@Au-Cys-FA nanocomposite. Fe_3O_4@Au-Cys-FA nanoparticles was synthesized using L-cysteine as a bi-functional linker The L-cysteine thiol (-SH) group links to the gold surface, and the amino ($-NH_2$) group condenses with the folic acid (FA) carboxylic acid group (–COOH) to form an amide bond [22]. Briefly, the red-brown Fe_3O_4@Au solution was adjusted to pH 10 using a 1% aqueous ammonia solution. L-cysteine (5 mL, 1 mM) was added to the Fe_3O_4@Au solution and the mixture stirred for 24 h at room temperature. During this phase, the thiol group on L-cysteine displaces the citrate moiety responsible for solvation of the nanoparticles. The Fe_3O_4@Au-Cys nanoparticles were separated using an external magnetic field, washed three times with water by magnetic decantation, and then dried in a desiccator.

Next, Fe_3O_4@Au-Cys (50 mg) was dispersed in 10 mL of CH_2Cl_2. Folic acid (0.1 g) was dissolved in 10 mL of DMSO, and N, N′ dicyclohexylcarbodiimide (DCC) (0.06 g) was added to the solution. The Fe_3O_4@Au-Cys dispersion and folic acid-DMSO solution were sonicated together in an ultrasonic bath for 30 min, and the solution then stirred at room temperature for 24 h. Fe_3O_4@Au-Cys-FA nanoparticles were separated using an external magnetic field, then washed three times with CH_2Cl_2, deionized water, and ethanol. After drying in a desiccator, Fe_3O_4@Au-Cys-FA nanoparticles were obtained as a red-brown powder.

2.3.4. Fabrication of the Fe_3O_4@Au-Cys-FA carbon-paste electrode

Fig. 1 illustrates the preparation of the sulfite biosensor. The Fe_3O_4@Au-Cys-FA/CPE device was prepared by thoroughly mixing graphite powder (0.950 g), Fe_3O_4@Au-Cys-FA nanoparticles (0.035 g), and PDMS (0.015 g) to a homogenous consistency using a mortar and pestle. The composite material was then heated to 60 °C for 30 min Immobilization of SOx was performed by dropping SOx solution (40 μL, 0.1 mg mL^{-1}), containing Cyt c (40 mg mL^{-1}), onto the composite. Cyt c was using as mediator between SOx and the electrode to obtain selectivity for sulfite determination. Mineral oil (30 μL) was then added and thoroughly mixed. The electrodes were fabricated in-house as described in the cyclic voltammetry section below.

2.4. Sample preparation for sulfite determination

Various brands of wines and pickled food samples, including mango, cabbage, and ginger were used for sulfite assay. Wine samples were filtered through a 0.25-micron cellulose membrane and diluted appropriately with phosphate buffer prior to sulfite determination. For pickled food samples, we used an extraction scheme described by Alamo et al. [26]. Briefly, samples were weighed in 50 g aliquots, and homogenized in phosphate buffer (50 mL) using a blender. The homogenized mixture was collected and filtered through a 0.25-micron cellulose membrane. Sulfite content was quantified by the standard addition method.

2.5. Method validation

Sulfite determination results obtained using the proposed FI-sulfite biosensor were compared to those obtained from a standard iodometric method [27,28]. An accurately measured sample volume (5.00 mL) was transferred into a 125 mL conical flask, and 5.00 mL of standard iodine solution was added. Excess iodine was titrated with standard sodium thiosulfate solution using starch as indicator. Titrations were carried out as quickly as possible, with the end point indicated by the formation of a light blue color.

Wine and pickled food samples were purchased from local supermarkets in Ubon Ratchathani province, Thailand. Five brands of wines (W1-W5) and three brands each of picked cabbage (C1-C3), pickled mango (M1-M3), and picked ginger (G1-G3) were used for sulfite assay using the developed amperometric biosensor. Sample aliquots were diluted 3 or 6 fold with phosphate buffer prior to analysis.

3. Results and discussion

3.1. Characterization of Fe_3O_4@Au-Cys-FA nanocomposites

Nanocomposite morphology was studied by TEM, SEM, and EDS. TEM samples were prepared by ultrasonic dispersion of the product in deionized water, and drying a drop of the resulting suspension on a copper grid. Fig. 2 shows TEM images of (A) synthesized Fe_3O_4 nanoparticles, and (B) Fe_3O_4@Au nanocomposites. Fe_3O_4 nanoparticles appear approximately spherical in shape with an average diameter of 11.5 ± 2.1 nm. After reduction of Au^{3+} to Au^0 on the Fe_3O_4 nanoparticle surface, the obtained nanocomposites appear much darker than the uncoated Fe_3O_4 nanoparticles (Fig. 2B) because of heavy atom effects [22,29]. The figure shows that the nanocomposite materials aggregate when dried [23,29,30]. The average Fe_3O_4@Au nanocomposite diameter is 14.1 ± 3.3 nm. The formation of Fe_3O_4@Au-Cys-FA was confirmed by SEM and EDS (Fig. 2C and D). Fig. 2C shows the micro-morphologies of the synthesized Fe_3O_4@Au-Cys-FA. SEM images of Fe_3O_4 and Fe_3O_4@Au (Supplementary Fig. S1) further suggests that the surface modification of Fe_3O_4 and Fe_3O_4@Au supports were successful. We used EDS to characterize the composition of the synthesized Fe_3O_4@Au–Cys-FA nanocomposites (Fig. 2D). The EDS spectrum reveals the presence of Fe, O, Au, and S atoms, and provides evidence for an attachment between Fe_3O_4@Au and folic acid via the thiol group on L-cysteine,

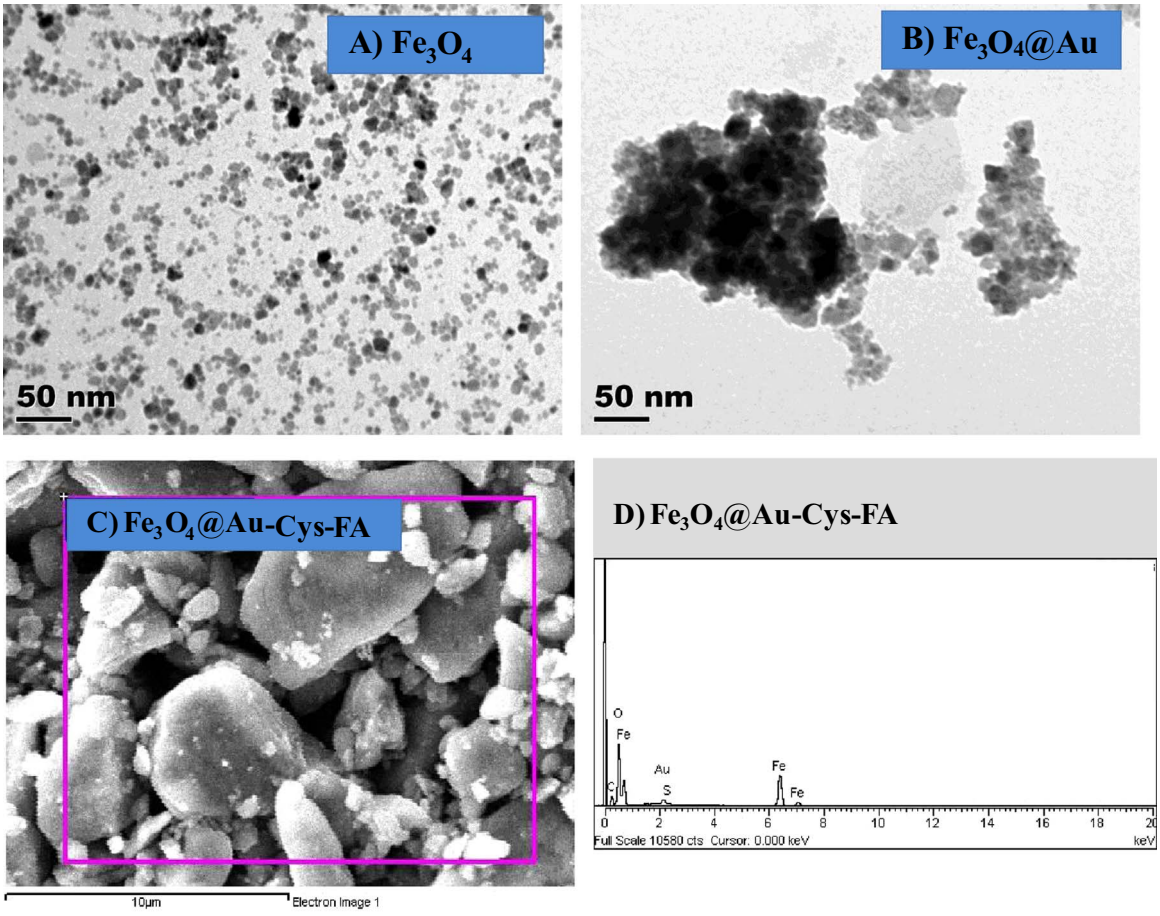

Fig. 2. TEM images of (A) Fe_3O_4 and (B) Fe_3O_4@Au. (C) SEM image of Fe_3O_4@Au-Cys-FA nanoparticles, and (D) the EDS spectrum of Fe_3O_4@Au-Cys-FA nanoparticles.

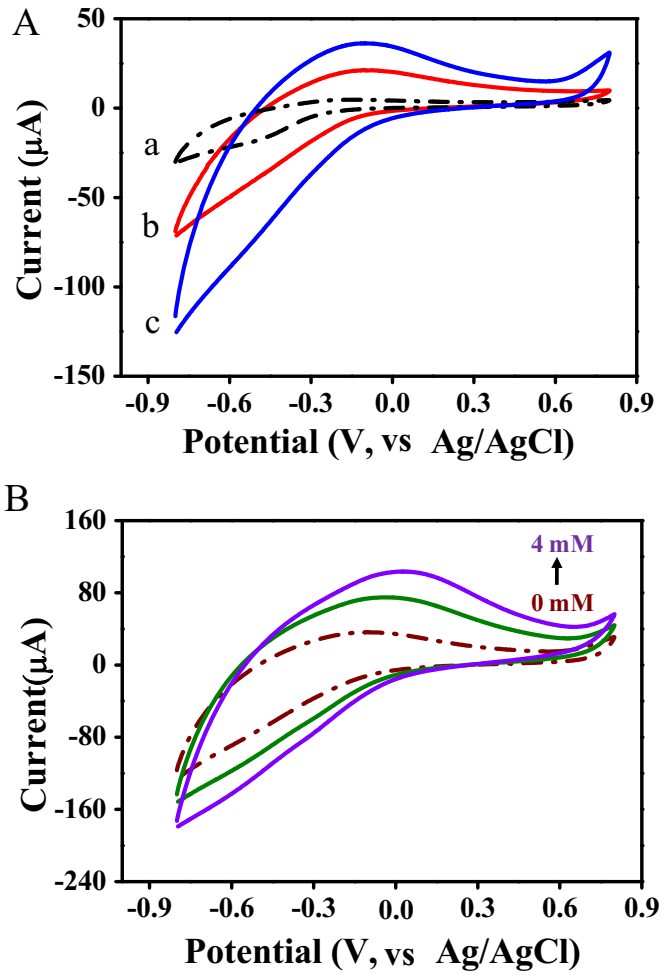

Fig. 3. (A) Cyclic voltammograms of (a) Fe_3O_4@Au/CPE biosensor, (b) Fe_3O_4@Au-Cys/CPE biosensor, and (c) Fe_3O_4@Au-Cys-FA/CPE biosensor, in 0.1 M PBS (pH 7) at a scan rate of 50 mV s^{-1}. (B) CVs of 0, 2, and 4 mM of sulfite on the Fe_3O_4@Au-Cys-FA/CPE biosensor, at a scan rate of 50 mV s^{-1}.

confirming the formation of Fe_3O_4@Au-Cys-FA [22].

3.2. Electrocatalytic behavior of the Fe_3O_4@Au-Cys-FA carbon paste electrode

Cyclic voltammetry (CV) is a useful technique to study the electrocatalytic behavior of nanomaterial-modified electrodes. The electrochemical behaviors of the carbon paste electrodes containing immobilized SOx were investigated using 0.1 M PBS as the supporting electrolyte. Fig. 3A shows cyclic voltammograms for (a) Fe_3O_4@Au/CPE, (b) Fe_3O_4@Au-Cys/CPE, and (c) Fe_3O_4@Au-Cys-FA/CPE. The Fe_3O_4@Au, Fe_3O_4@Au-Cys, and Fe_3O_4@Au-Cys-FA carbon-paste biosensors produce characteristic responses. The cyclic voltammogram for the Fe_3O_4@Au-Cys-FA modified carbon paste (Fig. 3A, curve c) shows the greatest current response, and exhibits the least negative peak potential when compared to the Fe_3O_4@Au and Fe_3O_4@Au-Cys CV plots. Therefore, we conclude that Fe_3O_4@Au-Cys-FA will provide the greatest enhancement in redox response and electrocatalytic activity.

Fig. 3B shows voltammograms obtained in the absence of sulfite (dotted line), and in the presence of 2 mM and 4 mM sulfite (solid lines) for the Fe_3O_4@Au-Cys-FA/CPE biosensor. Fe_3O_4@Au-Cys-FA/CPE biosensor exhibits quasi-reversible oxidation peaks at approximately −0.10 V versus Ag/AgCl. The oxidation peaks at approximately −0.10 V correspond to oxidation of H_2O_2 by enzymatic reaction between SOx and sulfite. The figure shows that increased sulfite concentrations induce apparent enhancements in the oxidation-reduction peak currents, because of increased H_2O_2 concentrations during enzymatic reaction. These oxidation peaks at approximately −0.10 V clearly demonstrate the Fe_3O_4@Au-Cys-FA/CPE catalytic properties. The electrochemical reactions associated with this amperometric biosensor may be a two-step process [31–33]:

$$SO_3^{2-} + O_2 + H_2O \xrightarrow{\text{sulfite oxidase}} SO_4^{2-} + H_2O_2 \quad (1)$$

$$H_2O_2 \xrightarrow{\text{CPE}} O_2 + 2H^+ + 2e^- \quad (2)$$

H_2O_2, the product from Eq. (1) was electrochemically measured at the sulfite biosensor (Fe_3O_4@Au-Cys-FA/CPE). Thus the amount of H_2O_2 produced in the SOx reaction can be used to indirectly determine sulfite level. To clarify that the observed catalytic oxidation current was due to the generated H_2O_2 by SOx, the effects of H_2O_2 on the non-enzymatic electrode based on CPE modified with Fe_3O_4@Au-Cys-FA were investigated. Cyclic voltammograms (Fig. S2d) show that the oxidation current based on $H_2O_2$from +0.35 V was observed at the non-enzymatic electrode. Furthermore, FIA grams obtained from amperometric measurement of the non-enzymatic electrode at +0.35 V (vs. Ag/AgCl), (Fig. S5) revealed that the oxidation peak currents increase linearly with increasing the concentration of H_2O_2.

In this work, a Nafion® film formed by drop casting provides a stable support to the Fe_3O_4@Au-Cys-FA/CPE biosensor. It is known that chemically inert ionomeric membrane of Nafion® can be mainly used to avoid influence negatively charged interferents such as ascorbate [34,35] in real samples. On the other hand, to clarify the acceptable permeable of the negatively charged of sulfite analyte to the Nafion® covered electrode surface is caused. Linear correlation between oxidation currents and the concentration of analyte obtained from CV and FIA experiments (Fig. 3B and Fig. S5) demonstrated that there are efficient diffusion shuttle between the Nafion® film and electrode surface. These results are in good accordance with the reports for the application of Nafion® coated electrode surfaces for determination of some anions including nitrite [36], sulfite [37] and the reduction of dioxygen to H_2O_2 [38].

The relationship between peak current and scan rate was examined at the Fe_3O_4@Au-Cys-FA/CPE. The results reveals that anodic and cathodic peak currents (μA) increase linearly with the square root of the scan rate ($\nu^{1/2}$ $s^{-1/2}$) over the scan range 0.05–0.40 V s^{-1}. Linear regression analysis provides r^2 values of 0.995, indicating a diffusion controlled quasi-reversible electrochemical process.

To investigate the effect of sulfite concentration over the range 0.5–10 mg L^{-1}, we calculated the apparent Michaelis-Menten constant (K_M^{app}) from a Lineweaver-Burk plot (data not shown) [23,39,40]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}C} \quad (3)$$

where I_{ss} is the steady-state current after addition of the substrate; I_{max} is the maximum current measured under saturated substrate conditions, and C is the bulk concentration of the substrate. The K_M^{app}value for the Fe_3O_4@Au-Cys-FA/CPE device is calculated as 2.00 mg L^{-1} (25 μM), indicating that our biosensor has a high affinity for immobilized SOx immobilization during H_2O_2 determination. This value for K_M^{app} is smaller than the value of 8.08 mg L^{-1}, seen for AuNPs-PEI, in which SOx is immobilized onto an Au electrode modified with gold nanoparticles capped with cationic branched poly(ethyleneimine) [41], and is comparable to reported values of 2.05 mg L^{-1} [40], 0.64 mg L^{-1} [42], and

1.60 mg L^{-1} [32] for SOx immobilized onto a gold-nanoparticle/chitosan/carbon nanotube/polyaniline composite modified Au electrode [40], gold-coated magnetic nanoparticles [42], and an ITO electrode modified with a PEI capped CdS quantum-dot [32], respectively. This result indicates that our biosensor provides good SOx electrocatalytic activity.

3.3. Amperometric detection in the FI system

3.3.1. Optimization

We investigated how the carbon-paste binder, applied potential, and flow rate, affect the sensitivity and stability of the sulfite determination in a FIA system. The CPE has a porous surface that contains a mixture of conducting graphite, catalyzing materials, and non-conducting mineral oil binder. PDMS oil has attractive properties for constructing carbon paste based chemical sensors [43] and biosensors [23,44,45]. Used in a glucose biosensor application, the oil provides an internal source of oxygen for enzymatic reaction [23,44,45]; the solubility of oxygen in PDMS is 45–50 fold greater than it is in water [46]. In the construction of a CPE for chemical sensing applications [43], use of a PDMS-mineral oil binder has been demonstrated to enhance the physical stability of the electrode, and facilitate the sealing the CPE to microfluidic devices [43].

The performances of the Fe_3O_4@Au-Cys-FA/CPE electrode using both mineral oil and a mineral oil-PDMS mixture as binder were compared using amperometry. Fig. 4A shows the amperometric responses to standard sulfite (20 μL, 10 mg L^{-1}) by the Fe_3O_4@Au-Cys-FA/CPE biosensor using (a) mineral and (b) mixed mineral oil-PDMS as binder. Sulfite oxidation peaks are clearly visible for the mixed mineral oil-PDMS binder (curve b), while the signal obtained from the mineral oil CPE was barely detectable (curve a). CPE sensitivity is much greater using the mixed binder than it is with mineral oil alone. The observed improvement in CPE sensitivity and physical stability in the presence of PDMS has been reported for other amperometric biosensors [43–45].

We investigated the optimal potential for amperometric detection at the Fe_3O_4@Au-Cys-FA/CPE device over a potential range from −0.10 to +0.80 V (Fig. 4B). Each datum represents the average of triplicate standard sulfite injections (20 μL, 10 mg L^{-1}). The modified CPE produces strong current responses at potentials between +0.20 and +0.35 V. The signal remains steady at potentials greater than +0.35 V. Maximum sensitivity occurred at an operating potential of +0.35 V (versus Ag/AgCl), and thus, we used this optimal voltage for amperometric detection.

To maximize sample throughput with satisfactory sensitivity, we varied flow rate over the range 0.2–1.8 mL min^{-1} (Fig. 4C). The signal response decreases with increasing flow rate. High flow rates normally result in a reduced response because of the shorter contact time between enzyme and analyte, as reported for other enzyme-based amperometric detection FIA systems [23,47,48]. However, sample throughput rapidly increases with increasing flow rate over the range 0.2–1.4 mL $min^{-1}$and remains constant over the range 1.4–1.8 mL min^{-1}. To provide a balance between sensitivity and sample throughput, we selected an optimal flow rate of 0.8 mL min^{-1}.

3.3.2. Analytical features

We investigated the analytical performance of the FI amperometric detection method with the developed sulfite biosensor. Under optimal conditions (operational potential, +0.35 V; carrier solution, 0.1 M PBS, pH 7; flow rate, 0.8 mL min^{-1}), FIA traces were obtained (Fig. 5A), and these were used to prepare a calibration curve. The figure illustrates a satisfactorily linear correlation from 0.1 to 200 mg L^{-1}. The regression equation is given by $y = 1.086x + 1.147$ ($r^2 = 0.998$), where y and x are the height of peak current (μA) and sulfite concentration (mg L^{-1}), respectively. The slope of the equation is corresponding to linear sensitivity of 1.086 μA $(mg)^{-1}$ L. The relative standard deviation (%R.S.D.) calculated from the current response among the sulfite concentrations in the calibration curve are ranged from 0.4–5.1% (Table S1) indicates good precision of developed technique. The calculated detection limit for the biosensor is 10 μg L^{-1} (3σ of blank). The limit of quantification is 34 μg L^{-1} estimated according to ISO11843 [15,49]. Sample throughput is 109 samples h^{-1}. Our Fe_3O_4@Au-Cys-FA/CPE shows good linearity over the concentration range, and a low limit of detection compared with previously reported amperometric sulfite biosensors (Table 1). The linear range of our biosensor is comparable with [14,51] or better than [15,31,40–42,50] other biosensors using immobilized SOx electrode. The developed biosensor offers a comparable [31,42,50] or

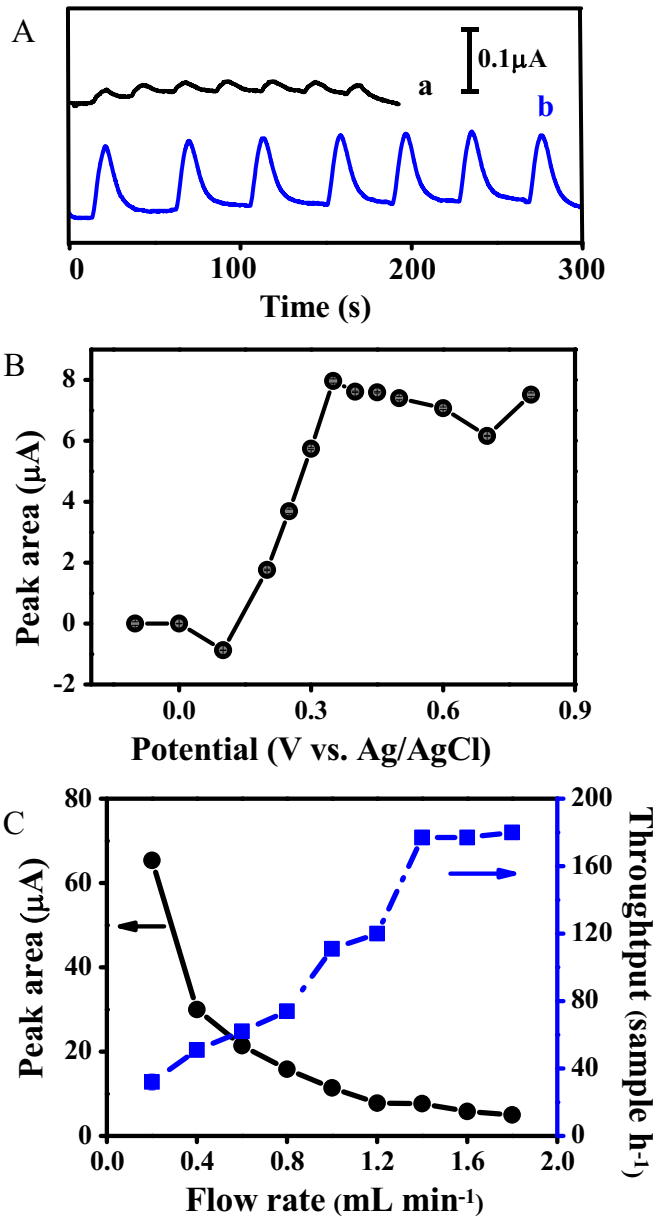

Fig. 4. (A) FIA grams comparing sulfite detection performances for mineral oil and mixed mineral oil-PDMS binders; sulfite, 10 mg L^{-1}; applied potential, +0.35 V; flow rate, 1.0 mL min^{-1}, ambient temperature (~25 °C). (B) Influence of applied potential on the biosensor response; sulfite, 10 mg L^{-1}; flow rate, 1.0 mL min^{-1}. (B) Effect of flow rate at Fe_3O_4@Au-Cys-FA/CPE on sulfite response and sample throughput; sulfite, 20 mg L^{-1}; applied potential,+0.35 V.

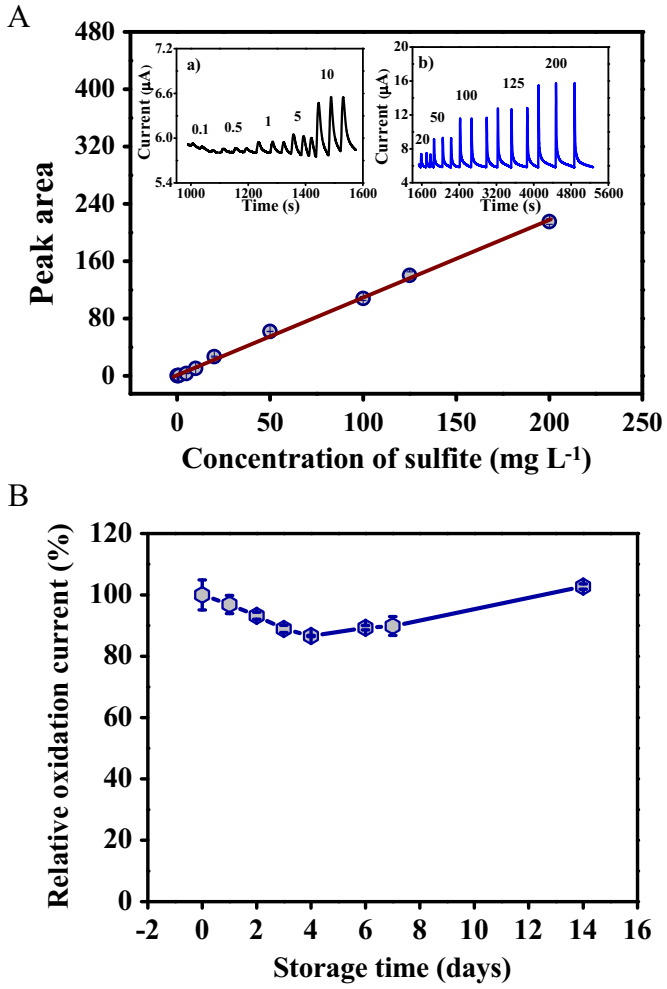

Fig. 5. Calibration plot of peak area of current signals versus concentration of sulfite as obtained from the FIA grams. The inset shows FIA grams for sulfite standards (a) 0.1–10 mg L^{-1} and (b) 20–200 mg L^{-1}, obtained as the averages of triplet injections.

lower [14,15,40] detection limit compared to other sensors of similar design. Moreover, the operating potential for our biosensor is lower than [40,50] or comparable to previous reports [14,15,31,42]. A low operating potential minimizes interferences from sample constituents. Major advantages of the Fe_3O_4@Au-Cys-FA/CPE over previously reported devices are ease of preparation and high stability. Our approach, immobilize SOx on the Fe_3O_4@Au-Cys-FA modified carbon-paste electrode provides in high electrode stability and improves biosensor performance toward sulfite detection.

Reproducibility and stability are important features for the practical use of the biosensors. In this report, we have demonstrated that the CPE provides good precision (%R.S.D.=3.1) for 20 μL injections (n=20) of 15 mg L^{-1} sulfite. Repeatability between successive tests was investigated using the same biosensor, and monitoring the current response of sulfite at an applied potential of +0.35 V (vs. Ag/AgCl). As shown in Fig. 5B, current responses of the novel biosensor remain close to the initial measurements. The current response of the biosensor retains 89% of the initial current response after repeated use for more than one week, and retains approximately 102% after 2 weeks when it was stored at 4 °C. These results suggest that the novel biosensor performs with satisfactory stability.

3.4. Interference study and application to real samples

To evaluate the selectivity of the proposed sensor, we examined the effects of interference, from selected inorganic ions and organic compounds, which are commonly present in sulfite samples. These compounds include sugar (glucose, sucrose, fructose, and maltose), ethanol, ascorbic acid, and various ions (NO_3^-, Cl^-, I^-, CH_3COO^-, and SO_4^{2-}). We studied the interference effects of foreign species on the FI signals obtained from standard 15 mg L^{-1} sulfite (Table 2). The tolerance was defined as the maximum concentration of the interfering substance required to cause a signal alteration greater than ±5%. Tolerances toward these compounds are 75 mg L^{-1} or greater, and negligible interference was observed during testing. We conclude that our proposed device provides good selectivity for the amperometric determination of sulfite.

The system was applied to the determination of the sulfite content of five wines (W1-W5) and pickled food extracts (G1-G3, M1-M3 and C1-C3) (Table 3). Sulfite determinations were performed by FIA with amperometric measurements under optimized conditions, using the standard addition method. Our results compare well with measurements obtained from the reference

Table 1
Comparison of analytical characteristics of the proposed sulfite biosensor with previously reported SOx-based biosensors.

Electrode type	material	K_M^{app} (mg L^{-1})	Potential (V)	Linearity range (mg L^{-1})	LOD (μg L^{-1})	Ref.
Au	AuNPs/CHIT/CNTs/PANI	2.05	+0.47	0.06–32	40	[40], batch
Au	PBNPs/PPY	–	+0.40	0.04–80	8	[50], batch
Au	Fe_3O_4@Au	0.64	+0.20	0.04–80	12	[42], batch
Au[a]	AuNPs-PEI	8.08	−0.10	0.04–0.43	–	[41], batch
ITO	PBNPs/PPY	–	+0.30	0.04–80	9.6	[31], batch
ITO[a]	PEI-CdS-QDs	1.60	−0.16	–	–	[32], batch
GC	MTF	–	−0.24	16–224	–	[51], batch
SPE	Cyt c	–	+0.30	4.1–750	4000	[14], batch
SPE	TTF	–	+0.20	0.79–6.61	480	[15], batch
CPE	Fe_3O_4@Au-Cys-FA	2.00	+0.35	0.1–200	10	This work, FIA

Au=gold electrode.
ITO=Indium tin oxide.
GC=glassy carbon.
SPE=screen printed electrode.
CPE=screen-printed electrode.
AuNPs=gold nanoparticles, CHIT=chitosan, CNTs=carbon nanotubes, PANI=polyaniline, PBNPs=Prussian blue nanoparticles, PPY=polypyrrole, MTF=Mercury thin film, Cyt c=Cytochrome C, TTF=tetrathiafulvalene, Fe_3O_4@Au=gold coated magnetic nanoparticles, PEI-CdS-QDs=polyethylenimine capped CdS quantum-dots, Fe_3O_4@Au-Cys-FA=folic acid-cysteine-conjugated gold-coated magnetite core shell.
[a] Using human sulfite oxidase (hSOx).

Table 2
Effects of foreign substances on the FIA signal, obtained from triplicate injections of sulfite standard (15 mg L^{-1}).

Foreign species	Results[a] (mg L^{-1})
Sugars	
Sucrose /$C_{12}H_{22}O_{11}$, fructose /$C_6H_{12}O_6$	1500
Glucose /$C_6H_{12}O_6$, maltose/$C_{12}H_{22}O_{11}$	750
Ions	
SO_4^{2-}/Na_2SO_4, Cl^-/NaCl, NO_3^-N/$NaNO_3$, CH_3COO^-/$NaCH_3COO$	1500
I^-/KI	150
Others	
Ethanol (CH_3CH_2OH)	750
Ascorbic acid ($C_8H_8O_6$)	75

[a] Greater than ±5% signal alteration is classified as an interfering condition.

Table 3
Comparison of sulfite determinations of wines (W1-W5) and picked food extracts (ginger; G1-G3, mango; M1-M3 and cabbage; C1-C3), between the developed Fe_3O_4@Au-Cys-FA/CPE biosensor and the reference iodometric method.

Samples	Sulfite (mg L^{-1} or mg kg^{-1})		%Relative difference
	Developed biosensor[a]	Reference method[a]	
W1	12.94 ± 0.05	12.77 ± 0.01	−1.35
W2	2.74 ± 0.01	2.69 ± 0.01	−1.78
W3	11.67 ± 0.02	11.42 ± 0.01	−2.14
W4	5.59 ± 0.09	5.38 ± 0.03	−4.05
W5	8.79 ± 0.04	8.74 ± 0.02	−0.63
G1	23.81 ± 0.04	22.85 ± 0.01	−4.21
G2	24.55 ± 0.03	23.52 ± 0.01	−4.38
G3	23.21 ± 0.02	22.18 ± 0.01	−4.67
M1	23.12 ± 0.04	23.52 ± 0.01	+1.72
M2	42.06 ± 0.02	43.68 ± 0.01	+3.71
M3	37.29 ± 0.03	36.96 ± 0.07	−0.90
C1	24.29 ± 0.05	24.19 ± 0.01	−0.40
C2	24.29 ± 0.10	24.86 ± 0.01	+2.29
C3	30.77 ± 0.02	29.57 ± 0.01	−4.06

[a] Average ± standard deviation of 3 measurements.

iodometric method. The relative differences between data obtained from the Fe_3O_4@Au-Cys-FA/CPE biosensor and the iodometric method range from 0.4–4.7%, and are in good agreement. Statistical analysis (paired *t*-test [52]) reveals that data from our developed biosensor are not significantly different to that from the reference method ($t_{observed}$ = 0.996, $t_{critical}$ = 2.161, 95% confidence). The results indicate that our sulfite biosensor is sufficiently accurate, and is suitable for the quantification of sulfite in the study samples.

4. Conclusion

In this work, we described a novel sulfite biosensor fabricated by immobilizing sulfite oxidase by chemisorption on the Fe_3O_4@Au-Cys-FA nanocomposite-modified carbon-paste electrode. The Fe_3O_4@Au-Cys-FA/CPE shows a significant improvement in biosensor performance when compared to the Fe_3O_4@Au/CPE and Fe_3O_4@Au-Cys/CPE devices. The biosensor produces oxidation peaks that correspond to oxidation of H_2O_2 by an enzymatic reaction between SOx and sulfite ion. Thus, we quantified sulfite by taking amperometric measurements at the Fe_3O_4@Au-Cys-FA/CPE biosensor using an in-house assembled flow injection system at +0.35 V. Enhancement of the electrode physical stability and sensitivity of the response was achieved using mixed PDMS-mineral oil as binder for electrode fabrication. Under optimal conditions, the proposed biosensor exhibits rapid sample throughput, with excellent sensitivity and selectivity toward sulfite quantitation. Moreover, the Fe_3O_4@Au-Cys-FA/CPE biosensor exhibits good stability in our flow system, and presents fair reproducibility for amperometric detection. The method was successfully applied to sulfite determination in wines and pickled food extracts.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.04.066.

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