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Sulfite oxidase biosensors based on tetrathiafulvalene modified screen-printed carbon electrodes for sulfite determination in wine

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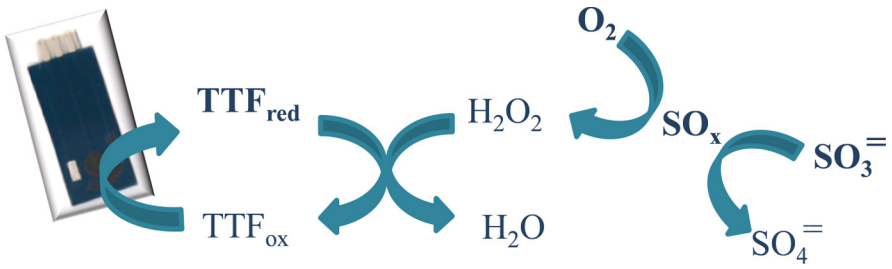
HIGHLIGHTS

- SOx has been cross-linked onto the surface of SPCEs modified with TTF.
- Sensitive and selective determination of sulfite has been performed.
- The developed procedure allows working at +200 mV, avoiding interferences.
- White and red wine samples have been successfully analyzed.

GRAPHICAL ABSTRACT



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ABSTRACT

Screen-printed carbon electrodes have been modified with tetrathiafulvalene and sulfite oxidase enzyme for the sensitive and selective detection of sulfite. Amperometric experimental conditions were optimized taking into account the importance of quantifying sulfite in wine samples and the inherent complexity of these samples, particularly red wine. The biosensor responds to sulfite giving a cathodic current (at +200 mV vs screen-printed Ag/AgCl electrode and pH 6) in a wide concentration range, with a capability of detection of 6 μM ($\alpha = \beta = 0.05$) at 60 °C. The method has been applied to the determination of sulfite in white and red samples, with averages recoveries of 101.5% to 101.8%, respectively.

1. Introduction

Sulfite plays a very important role during production and storage of food and drinks, including wine [1]. Being naturally produced during winemaking, sulfite is also usually added to the product to limit the microbial growth [2] and to control the taste, texture and colour attributes of the final product [1]. However, sulfite has been reported to cause adverse health effects, associated to lung diseases, which have pointed out the need of control its amount in food and drinks [1,2].

Electrochemical techniques have been traditionally employed for detecting sulfite, using different electrodes. The typical problems associated to these procedures have been the fouling of electrodes, which lead to a loss in sensitivity, and the lack of selectivity due to the large overpotential applied to monitor the oxidation of sulfite. In order to improve both figures of merit, electrodes can be modified by metal complexes or biological agents [1]. Generally, biosensors have been developed using sulfite oxidase (SOx) enzymes immobilized onto different electrodes, such as gold, platinum or carbon (Table 1). Sulfite measurement mechanism by these biosensors is based on the well-known SOx-catalyzed

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Table 1
Analytical characteristics of amperometric SOx based biosensors for sulfite detection in wine.

Support for immobilization	Mediator	SOx immobilization procedure	pH	Working temperature	Working potential	Calibration range	Wine sample	Reference
Vitreous carbon	TCNQ/TTF	Encapsulation by dialysis membrane	8.5	Room	0 mV	0–0.5 mM	White	[13]
Platinized glassy carbon with polytyramine	–	Covalent	6	–	+500 mV	2–300 μM	White	[8]
Pt with PPy film		Entrapment	7	–	−700 mV	0.9–400 μM	White	[3]
PASA modified Au	Cyt c	Adsorption	8.5	–	+100 mV	1–60 μM	White	[14]
SPCEs	Os-polymer	Cross-linking	8.5	–	−100 mV	0.5–100 uM	White	[2]
AuNPs/CHIT/MWCNT/PANI/Au		Covalent	7	35 °C	+600 mV	0.75–400 uM	Not specify	[9]
PBNPs/PPy/ITO		Adsorption	8.5	35 °C	+300 mV	0.5–1000 uM	Red	[10]
Fe_3O_4@GNPs/Au		Covalent	8.5	35 °C	+200 mV	0.5–1000 uM	White, red	[11]
PBNPs/PPy/Au		Adsorption	8	30 °C	+400 mV	0.5–1000 uM	White, red, rose	[12]
SPCEs	TTF	Cross-linking	6	40 °C	+200 mV	9.9–82.6 μM	White, red	This work

AuNPs gold nanoparticles, CHIT chitosan, Fe_3O_4@GNPs gold coated magnetic nanoparticles, ITO indium tin oxide, MWCNTs multiwalled carbon nanotubes, TCNQ tetracyanoquinodimethane, TTF tetrathiafulvalene, PANI polyaniline, PASA polyanyline sulfonic acid, PPy polypyrrole, PBNPs prusian blue nanoparticles,)

reaction, in which the molybdenum active sites in SOx undergo oxidation and reduction states during the catalytic oxidation of sulfite to sulfate [3]. The detection of sulfite can be achieved by either detecting the direct SOx-electrode communication [4–7], the depletion of oxygen [3] or the generation of peroxide [8–12], being the latter the simplest approach. Its main drawback is the large overpotential that is usually required. Thus, electron-transfer mediators have been introduced to achieve the selective redox determination of sulfite [1]. The analytical signal was then based on the regeneration of the mediator [2,13,14] or on the catalysis of peroxide conversions such that the operating potential of the biosensor can be minimized [1].

The use of screen-printed carbon electrodes (SPCEs) would simplify the use of these electrochemical biosensors, avoid the fouling of electrodes due to their disposable character and highlight the possibility of carrying out decentralized assays [1,2,15,16]. Moreover, these devices allow using binding matrices containing the electron-transfer mediator to be also screen-printed, which results in electrochemical advantages such as fast response, low background currents, operational and storage stability [17]. Taking into account the low solubility of tetrathiafulvalene (TTF) in water and its low redox potential, it has been reported as an optimum mediator in order to be screen-printed for working in aqueous solutions [18–21]. Consequently, the aim of this work has been the development of electrochemical biosensors using TTF modified SPCEs ($SPC_{TTF}Es$) with cross-linked SOx (SOx–$SPC_{TTF}Es$) for the detection and quantification of sulfite in white and red wine samples.

2. Experimental

2.1. Chemicals and reagents

Silver (5029 conductor paste, DuPont (UK) Limited, Bristol, England), Ag/AgCl (Electrodag 6037 SS, Acheson Colloiden, Scheemda, The Netherlands), carbon (C200802P2, Gwent Electronic Materials, Torfaen, UK) and dielectric (D2071120D1 Gwent Electronic Materials, Torfaen, UK) inks were used in the fabrication of the based transducers.

All reagents used were of analytical-reagent grade. Milli-Q water (Millipore, Bedford, MA, USA) was used for preparing aqueous solutions.

A 50 mM phosphate buffer pH 6 (Panreac, Barcelona, Spain) containing 100 mM of KCl (Merck, Darmstadt, Germany) was used as supporting electrolyte solution. pH values were adjusted using a 2 M solution of NaOH (JT Baker, Deventer, The Netherlands).

Recombinant human SOx purified from *Escherichia coli* (0.5 U μL^{-1}), sodium sulfite anhydrous and TTF were obtained from Biolan Microbiosensores (Parque Tecnológico de Vizcaya, Zamudio, Spain), Panreac (Barcelona, Spain) and Acros Organics (Geel, Belgium), respectively. Bovine serum albumine (BSA) and glutaraldehyde (GA) were provided by Sigma–Aldrich (Steinheim, Germany).

2.2. Biosensors construction

$SPC_{TTF}Es$ were homemade built using a DEK 248 printing machine (DEK, Weymouth, UK). These transducers consisted of three screen-printed electrodes deposited onto polyethylene terephthalate films (HiFi Industrial Film, Dardilly, France). The different inks were screen-printed and cured according to the manufacturer's specifications [22]. The working electrode ink was prepared by thoroughly mixing carbon ink with TTF (3% v/w) and immediately screen-printed. Fig. 1 shows an image of the SPCEs configuration used (Working area, 15.90 mm^2).

SOx was cross-linked onto SPCEs and $SPC_{TTF}Es$, using BSA as non-active protein in order to prevent the loss of enzymatic activity that can cause the cross-linker GA [23,24]. Different experiments were carried out using different concentrations of GA, BSA and SOx in order to obtain the maximum reduction current. The optimum one was achieved by subsequently dropping 1 μL of a BSA solution (3% w/v), 3 μL of a GA solution (5% w/v) and 2 μL of SOx onto the working electrode surface. The mixture was left to react 60 min at 4 °C and then, washed with phosphate buffer and stored at 4 °C when not in use.

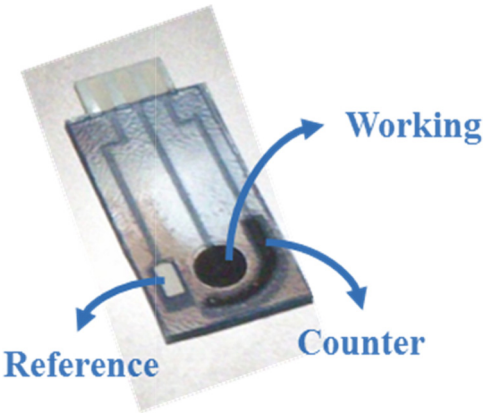

Fig. 1. SPCEs image.

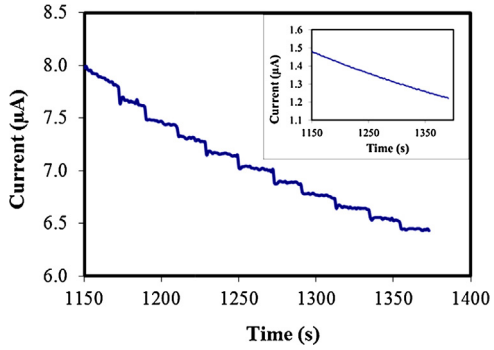

Fig. 2. Amperogram recorded using a SOx–$SPC_{TTF}E$ at +200 mV vs a screen-printed Ag/AgCl electrode, in a 5 mL supporting electrolyte solution pH 6, at 40 °C. Each addition corresponds to 100 μL of a 1 mM solution of sulfite. Inset: Amperogram recorded using a $SPC_{TTF}E$ in the same conditions.

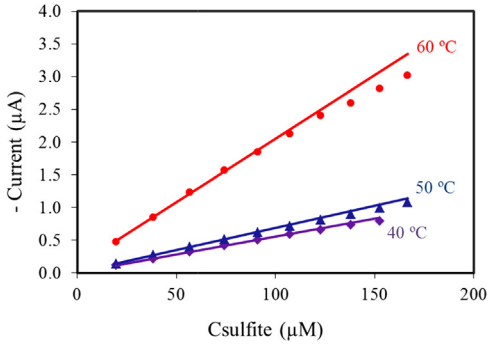

Fig. 3. Influence of the working temperature in the determination of sulfite. Calibration curves recorded using SOx–$SPC_{TTF}Es$ at +200 mV vs a screen-printed Ag/AgCl, in supporting electrolyte solutions at 40 °C (♦), 50 °C (▲) and 60 °C (●). Sulfite concentration range from 19.9 to 196.1 μM.

2.3. Experimental procedure

Amperometric measurements were carried out in a batch system, with constant stirring, using a PalmSens® portable electrochemical potentiostat with the PS Trace program (PalmSens® Instruments BV, Houten, The Netherlands). All measurements were made in a cell containing 5 mL of supporting electrolyte solution at 40, 50 or 60 °C. A potential of +200 mV vs screen-printed Ag/AgCl electrode was applied and the corresponding sample, thermostated at working temperature, was added after reaching a stable baseline.

3. Results and discussion

Sulfite biosensors were developed by cross-linking SOx onto SPCEs and $SPC_{TTF}Es$. To evaluate the catalytic activity of SOx, amperograms were registered at different working potentials. SOx oxidizes sulfite to sulfate, generating hydrogen peroxide. These enzymatically-generated products are oxidant enough to oxidize TTF, which is electrochemically regenerated [1,18–20]. Thus, a cathodic current was observed when SOx–$SPC_{TTF}Es$ were at least polarized at a potential of +200 mV vs screen-printed Ag/AgCl electrode (Fig. 2). This working potential was selected as optimum since low working potentials may be preferably used for the analysis of samples potentially containing interfering compounds, e.g., red wine [17]. Furthermore, no direct reduction current was observed at +200 mV vs screen-printed Ag/AgCl electrode using SOx–SPCEs or at $SPC_{TTF}Es$ modified with GA and BSA, but without SOx, in presence of a 20 μM sulfite solution. This fact highlights the relation between sulfite concentration and the cathodic current registered at SOx–$SPC_{TTF}Es$.

The choice of the most appropriate working pH was carried out by comparing the analytical signal of the biosensor for a 20 μM sulfite solution with that achieved for a wine sample with a similar amount of sulfite, using SOx–SPCEs. A huge cathodic current was recorded at +200 mV vs screen-printed Ag/AgCl electrode for the wine sample in relation to the sulfite solution at pH 5 and 7, which can be attributed to the presence of sulfite and different interferences. However, the reduction currents recorded at pH 6 were of analogous magnitude for both solutions (Table 2). The level of interferences due to the compounds present in the wine sample was minimized at pH 6, so this value was chosen as the working pH.

Similarly, the influence of temperature on the maximum response current of the biosensor was evaluated [9–12,25]. Although temperature accelerates enzyme-catalysed reactions, proteins can be denaturalized from certain temperature. Above the optimum temperature, increasing the reaction rate due to temperature is offset by the loss of catalytic activity due to thermal denaturation, and the enzyme activity rapidly decreases until annulled. As it can be seen in Fig. 3, no significant differences are observed at 40 and 50 °C, but the response of the biosensor considerably increases at 60 °C. Thus, the performance of the developed biosensor for sulfite detection and quantification was analysed at 40 and 60 °C. With this aim, several calibration curves were performed, under optimum conditions, using SOx–$SPC_{TTF}Es$ by successive additions of a sulfite solution. The parameters of these regressions were optimally evaluated using the Progress program [26], which builds least median square (LMS) regressions in order to detect the anomalous points. Once, those points were removed from the calibration set, ordinary least square (OLS) regressions were built with the remaining points. This OLS regression provides correct assessment of the slope (sensitivity) and the calibration constant term, both being important for judging the quality of calibration and from this, the analytical method. The slopes of these calibration curves were used to estimate the reproducibility (inter-sensors) of both procedures in terms of relative standard deviation (RSD). It can be seen in Table 3 that the developed biosensor can be used with enough precision for the determination of sulfite in solution at both temperatures, especially considering that are disposable biosensors. Its capability of detection was estimated for a probability of false positive (α) and negative (β) of 0.05 [27,28]. The minimum detectable net concentration computed was 4.27 ± 0.27 μM ($n = 3$) and 1.08 ± 0.23 μM ($n = 3$), at 40 and 60 °C, respectively. Since these values are below the lowest calibration standard, 9.9 μM at 40 °C and 6 μM at 60 °C, the latter were taken as average capability of detection from an analytical point of view.

Last, the storage stability of the biosensor was evaluated for different biosensors manufactured the same day and stored at 4 °C

Table 2
Performance of the developed SOx–$SPC_{TTF}Es$ biosensors for the quantification of sulfite at different pH.

	Analytical signal of a 20 μM sulfite solution (nA)	Analytical signal of a wine with a 21 μM sulfite content (nA)
pH 5	160	660
pH 6	330	340
pH 7	170	590

Table 3
Performance of the developed SOx–$SPC_{TTF}Es$ biosensors for the quantification of sulfite at different temperatures.

SOx–$SPC_{TTF}Es$	40 °C	60 °C
Working potential (mV)	+200	+200
Calibration range (μM)	9.9–82.6	6.0–56.6
Reproducibility (% RSD)	1.2 ($n = 3$)	0.7 ($n = 3$)
Capability of detection, $\alpha = \beta = 0.05$ (μM)	9.9	6.0

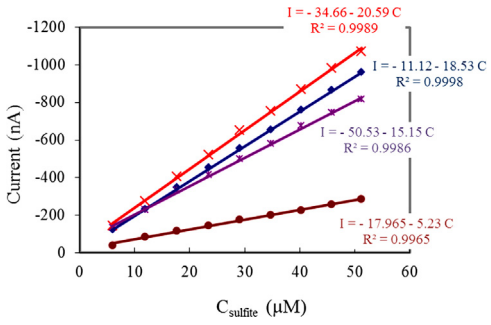

Fig. 4. Evaluation of the effect of the storage time on the sensitivity of SOx–SPC_{TTF}Es. Calibration curves recorded in the sulfite concentration range from 5.9 to 51.2 μM, under the optimum conditions, using four different SOx–SPC_{TTF}Es. (×) Calibration curve recorded the day that the biosensor was built, (♦) calibration curve recorded one month after, (*) calibration curve recorded 2 months after, (●) calibration curve recorded 3 months after.

until use. It was found that the response of the biosensor under the optimum working conditions was stable over 2 months after it was built (Fig. 4).

Taking into account the great performance of SOx–SPC_{TTF}Es, it was checked their viability in the determination of the content of sulfite in samples of white (1.09 mM) and red (1.04 mM) wines, using the method of standard additions. Amperograms were recorded under the optimum conditions and at 40 °C, due to the lower energy cost that involves. A volume of 100 μL of wine was added into the electrochemical system once a stable baseline was reached. Then, successive additions of a sulfite solution were performed in the concentration range from 19.9 to 166.7 μM.

The concentration of sulfite found was [1.10 ± 0.03] mM (n = 3, α = 0.05, RSD = 1.9%, average recovery = 101.5%) in the case of a white wine sample and [1.06 ± 0.05] mM (n = 3, α = 0.05, RSD = 4.1%, average recovery = 101.8%) for a red wine sample decolorized with active carbon (140 mg of active carbon per 1 mL of red wine). These results match up with the obtained values using segmented continuous flow analysis with colorimetric detection, given by the official laboratory of Oenological Station of Haro (La Rioja, Spain), accredited by EN/ISO 17025.

Since only a few SOx based biosensors have been applied to the analysis of sulfite in red wine samples with good recoveries (Table 1), these results highlight the great performance of the developed biosensor. Taking into account the simple fabrication process of SOx–SPC_{TTF}Es and their disposable nature, this methodology represents an attractive alternative to be routinely used at a winery.

4. Conclusions

A simple and effective electrochemical biosensor with good analytical properties such as fast response, long-time stability, and good detection range has been developed for the determination of sulfite. The modification of SPCEs with TTF and SO_X clearly improves the sensitivity for the analysis of this compound, exhibiting a linear response to sulfite in a wide concentration range. Its capability of detection, estimated for a probability of false positive (α) and negative (β) of 0.05, slightly improves from 9.9 to 6 μM when working at 60 °C. This easy modification procedure of SPCEs led to the amperometric determination of sulfite at +200 mV vs a screen-printed Ag/AgCl electrode. Since interferences are avoided at this low potential, the developed biosensor can be successfully applied to the determination of sulfite in white and red wines.

Acknowledgments

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