Determination of Sulfite in White Wine by Amperometric Flow Injection Analysis with an Immobilized Sulfite Oxidase Reactor

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A continuous-flow sensor, in which sulfite oxidase (SOD) was immobilized on CNBr-activated Sepharose, was developed for the determination of sulfite in white wine. Hydrogen peroxide produced by the enzyme reaction was monitored with a platinum electrode, which was covered with a dialysis membrane. The response of the sensor was linear in the range of 1 ~ 10 ppm sulfite, with a correlation coefficient of 0.999. The relative standard deviation for 10 injections was 2.3 % at the 5 ppm sulfite level. The gelatin coagulation procedure was introduced for the removal of interfering substances in wine such as polyphenol compounds. The method was applied to automatic sample pretreatment.

Sulfite is commonly used as a preservative in foods and in the pharmaceutical industry. It is added to food products to inhibit bacterial growth and to prevent oxidation. Its potential toxicity, however, makes its determination essential in control laboratories.

Various methods1 ~3) have been applied to the determination of sulfite and sulfur dioxide in solution, such as spectrophotometric determination with pararosaniline, titration with iodine and acid/base titration after oxidation. These methods are relatively time consuming, and some of them require a distillation procedure and also need relatively large amounts of samples. Consequently, a fast, simple method is required for the determination of sulfite.

Flow injection analysis (FIA) can be valuable in quality control because of its simplicity and the high sampling frequency, and it has also been applied to the determination of sulfite/sulfur dioxide with spectrophotometric,4 ~5) electrochemical,6 ~8) and chemiluminescence detection.9) On the other hand, an immobilized enzyme probe offers a rapid and reliable means of determining many compounds, and several enzyme10 ~12) or organella13) probes for sulfite/sulfur dioxide have been described, but only one has been applied to food samples.11) The combination of an enzyme probe and the flow injection analytical method constitutes a powerful technique for the determination of sulfite/sulfur dioxide in white wine.

Sulfite oxidase (EC 1.8.3.1) catalyzes the reaction shown in eq. (1).

\[
\text{SO}_3^{2-} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{Sulfite oxidase}} \text{SO}_4^{2-} + \text{H}_2\text{O}_2
\]

(1)

The hydrogen peroxide produced is monitored amperometrically by use of a platinum anode covered with a dialysis membrane. The measured current is proportional to the concentration of hydrogen peroxide produced and so to the concentration of sulfite in the sample solution. While developing the enzyme probe for the determination of sulfite and applying it to wine samples, the authors encountered severe interference by some components of wine.

In this study, sulfite was determined by use of sulfite oxidase immobilized on CNBr-
activated Sepharose incorporated into a flow-injection manifold. Automated pretreatment for the removal of interfering substances is also described.

**Materials and Methods**

*Reagents.* Sulfite oxidase (SOD, EC 1.8.3.1, from chicken liver, product No. S 0263) was obtained from Sigma Chemical Co. (Mo. U.S.A.). Sodium bisulfite, reagent grade, was obtained from Wako Pure Chemical Industries, Ltd. (Osaka), and used as a sulfite standard. CNBr-Activated Sepharose 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of reagent grade and were used without further purification.

Preparation of SOD immobilized on CNBr-activated Sepharose. SOD (30 units) was immobilized on CNBr-activated Sepharose (0.2 g dry weight) as reported previously, except than 0.1 m phosphate buffer (pH 8.0) was used as the coupling buffer and the material was packed into a glass tube (2 mm i.d., x 10 cm).

*Flow system.* A schematic diagram of the flow system is shown in Fig. 1. The working buffer was 0.1 m phosphate buffer (pH 8.0), unless otherwise specified. The main flow line was nearly the same as that described previously. The flow rate of the working buffer was 1.3 ml/min. The sample flow line included an automatic sample pretreatment device. A wine sample and 1% gelatin solution were mixed using micro-tube pump P1 at confluence point A and the colloidal coagulate produced in the solution was passed through a line filter (LF). The treated wine sample flowed into the working buffer line at confluence point B and was mixed thoroughly in the mixing coil (MC, 5 m-long, 1 mm i.d.). The flow rates of pump 1 (P1) and pump 2 (P2) were set so that the dilution ratio became about 1:20. Thus, the wine sample was pretreated to eliminate interfering substances (see "Removal of Interfering Substances" below) and diluted about 20-fold. The quadrangle, dot-dash-line, in Fig. 1 shows another stream line for the measurement of the blank value. The blank column was the same size as the enzyme column and contained Sepharose without the immobilized enzyme. The part of the apparatus represented by the quadrangle, dot-dash-
line, was only used when wine analysis was performed. In this case, the responses from the blank column were subtracted from those from the SOD column. The sample loops were set so as to be 240 μl each. The temperature of the immobilized SOD reactor and blank column was controlled at 25 °C. Amperometric measurements were made with a laboratory-made multichannel potentiostat, which was described previously.14) Other instruments in the system were the same as those in our previous work.14)

Flow-through Cell and Electrode. The three-electrode cell with a platinum working electrode used is shown in Fig. 2. The working electrode was a platinum wire (1 mmφ) with an area of 7.85 x 10⁻³ cm² and was welded to Cu-wire. The working electrode was covered with a cellulose dialysis membrane (Union Carbide Corp.), which was held in place with an O-ring. The counter electrode was a glassy carbon rod (5 mmφ, 5 mm-long), which was electrically connected to the Cu-wire via mercury. The cell was made water-tight by means of two O-rings, one of which was set in the cell body and the other on the working electrode, as shown in Fig. 2. The volume of the cell was about 380 μl. Electrical contact between the solution in the cell and the reference electrode (Ag/AgCl) was achieved through a potassium chloride-agar bridge via sintered glass. The potential of the working electrode in the three-electrode system was set so as to be +0.50V vs. Ag/AgCl, unless otherwise mentioned.

Procedures. A standard solution, 10,000 ppm level, of sodium bisulfite was prepared daily in deionized water. Standardization of the solution was performed by the iodometric method recommended by the AOAC, method 10.086.15) The solution was diluted to 1~10 ppm or 20~200 ppm with the assay buffer containing 0.1% (v/v) glycerol or 0.3% (w/v) fructose prior to analysis. Solutions of 1~10 ppm were used to investigate the optimum conditions for the sensor system and ones of 20~200 ppm were used to confirm the efficiency of the automatic sample treatment and dilution system.

F-kit Method. An F-kit (sulfite, test for the enzymatic determination of sulfurous acid ("total SO₂") in food-stuffs, No. 725854) was purchased from Boehringer Mannheim GmbH. The analysis was performed according to the manufacturer's manual. The method was also described in the literature.16)

Results and Discussion

Buffer and pH

Various kinds of buffer were investigated as to the sensor response of the immobilized SOD reactor. For the determination of sulfite in wine, buffer solutions containing 10 ppm sulfite and 1% ethanol were used as artificial sample solutions, corresponding in composition to 10-fold diluted ordinary wine. Phosphate buffer showed a greater response than the following three buffers when the same concentration (0.1 m) and the same pH (8.0) were used. The order of the magnitudes of the responses was 100 (phosphate), 94 (carbonate), 87 (pyrophosphate) and 60 (borate). The effect of pH on the response was studied from pH 6.0 to 9.0, using phosphate buffer. The results are shown in Fig. 3. The figure also shows the blank values. As can be seen in Fig. 3, the greatest response of the sensor and the lowest blank value were obtained at pH 8.0. The pH maximum for immobilized SOD shifted to the acidic side by 0.6 pH units, compared to that for the solubilized enzyme (pH 8.6).17) Therefore, phosphate buffer of pH 8.0 was used for further investigations.

Applied potential

In order to determine the optimum applied potential, the effect of the applied potential on the electrode response of 100 μM hydrogen peroxide and that of 5 ppm sulfite in 1% ethanol were investigated. In this experiment, the immobilized SOD reactor was omitted. The applied potential was changed from +0.40 V to +0.65 V vs. Ag/AgCl and the oxidation current was measured, the blank

Fig. 3. Effect of pH on the Response (●) and Blank Value (○).
column being set in the stream line, and each sample being directly introduced into the sample loop and injected. Amperometric monitoring of hydrogen peroxide is usually performed at an applied potential of $+0.60 \text{ V} \sim +0.70 \text{ V}$. As shown in Fig. 4, a plateau region was obtained from $+0.50 \text{ V}$ to $+0.60 \text{ V}$ for hydrogen peroxide. On the other hand, Sawyer et al.\textsuperscript{18} have reported that the anodic half wave potential of sulfite in neutral medium at a platinum electrode was ca. $+0.5 \text{ V}$ vs. SCE and a plateau region was obtained at ca. $+0.7 \text{ V}$ vs. SCE. In this study, a small current due to sulfite oxidation was observed at the applied potential of $+0.50 \text{ V}$, and the current response gradually increased with increasing applied potential from $+0.50 \text{ V}$ to $+0.65 \text{ V}$. In general, a high applied potential tends to produce interfering currents, because of the oxidation of other components in the samples. To avoid this difficulty, the applied potential selected for the present system was the smallest possible with which hydrogen peroxide produced the exact current response. The optimum applied potential, then, was judged to be $+0.50 \text{ V}$ and this was used throughout the following experiments.

**Flow rate**

Figure 5 shows the effects of the flow rate on the sensor response and the baseline reversion time (95% reversion). The response rapidly increased with increasing flow rate up to 0.45 ml/min, then gradually decreased and finally approached a plateau at a flow rate greater than 0.80 ml/min. A right-downward curve was obtained for the baseline reversion time. The choice of flow rate involves a compromise between sensitivity and sample output rate. A flow rate of 1.3 ml/min was used in this experiment, considering the relatively high response and the short sample output time.

**Enzyme amount, temperature and column length**

The effect of the amount of SOD on the sensor response is shown in Fig. 6. The response rapidly increased up to 150 U/g-dry Sepharose and became nearly constant above this amount. The effect of the temperature on the sensor response was investigated in the range of 15$\sim$40°C. A linear increase in response with increasing temperature up to 35°C was observed, while a serious decrease (only 9% of the response at 35°C) was observed at 40°C. Masoom and Townshend reported an increase in response with increasing temperature up to 50°C with Boehringer SOD.\textsuperscript{10} Smith, on the other hand, reported a temperature effect only up to 35°C with Sigma SOD.\textsuperscript{11} While the temperature behavior of the enzyme

![Fig. 4. Effect of the Applied Potential on the Response of Hydrogen Peroxide (●) and on the Direct Response of a Sulfite Solution Containing 1% Ethanol (○) without the Enzyme Column.](image)

![Fig. 5. Effect of the Flow Rate on the Sensor Response (○) and Baseline Reversion Time (●).](image)
will differ with the manufacturer, it is safe to conclude that the suitable temperature range is 20~35°C. The column length affected the sensor response; the response tended to increase with an increase in length. Because Sepharose 4B is a soft gel (highly expansive) and has a relatively poor flowing property in spite of the high efficiency of enzyme immobilization, a column of 10 cm-long was used in consideration of the low compression of the column.

**Calibration, reproducibility and long-term stability**

A typical calibration curve showed a linear relationship between the sensor response and the sulfite concentration over the range of 0.1~10 ppm, with a correlation coefficient of 0.999, under the optimum conditions. The relative standard deviation for 10 replicate injections was 2.3% at the 5 ppm level. The operational stability of the immobilized SOD reactor under the storage condition of 5°C was relatively good. The sensor response remained at the original value over 20 days and 67% of the original value remained after 45 days at the 5 ppm sulfite level.

**Removal of interfering substances**

When sulfite in wine was analyzed with a bare platinum electrode combined with the immobilized SOD reactor, severe interference by certain components in the wine was observed. The dialysis membrane used to cover the platinum electrode decreased the interference to 12% of the original value. The effectiveness of the dialysis membrane was not sufficient for wine determination, so additional treatments were investigated. Wine, especially red wine, contains relatively large amounts of polyphenol compounds. The interference was 10 to 20-fold greater in red wine than in white wine, and, consequently, the interfering substances were supposed to be polyphenol compounds such as tannins. Moreover, it was feared that tannins may cause inactivation of immobilized SOD and may spoil the flowing property of the SOD reactor. Some efforts were made to remove the interfering substances. The combined use of bovine serum albumin and ferrous ions in appropriate amounts, followed by filtration, decreased the interference to about 25% (75% removal). Polyvinylpolypyrrolidone, which is a recommended compound for the decolorization of colored juice, reduced the interference to 30% (70% removal). These procedures also reduced the sulfite content to a great extent. Gelatin was effective as to the removal of the interfering substances in wine. The treatment of wine (4 ml) with 2.5% gelatin (1 ml), followed by gentle mixing and filtration, reduced the interference to 50%, and no removal of sulfite was observed. To confirm the non-removal of sulfite by the treatment, a simulated wine comprising 10% ethanol, 100 ppm sulfite, 300 mg/l tannin, 8 g/l glucose and 14 g/l fructose was used to determine the sulfite content. After treatment of the simulated wine with gelatin, the solution was filtered and diluted 10-fold with phosphate buffer, and the resulting solution was injected into the sulfite determination system. The recovery of sulfite was 100% when a small interference signal was subtracted from the measured signal. The overall elimination efficiency using a dialysis membrane and the gelatin treatment was about 94% for white and red wine. This result indicates that, if the amount of the interfering
substances is relatively low, the gelatin treatment is useful for removing them.

*Automatic sample treatment* The efficiency of the sample pretreatment and dilution was investigated. A wine sample and 1% gelatin were transferred at the flow rate of 0.8 ml/min, and the resulting coagulate was passed through a line filter (flow rate, 1.6 ml/min), as shown in Fig. 1. The buffer solution was carried by a 9-channel micro-tube pump at the flow rate of 1.5 ml/min. The flow rate at confluence point B was about 15 ml/min. Five meters of mixing coil was required for uniform dilution of the sample. The dilution ratio was about 1 : 20. The rate of the sample exchange was less than 2 min/sample with this system. The filter paper (Advantec No. 2) in the line filter could be used for more than 50 samples without exchange. The calibration curve showed a linear relationship between the sensor response and the sulfite concentration over the range of 20 ~ 200 ppm, with a correlation coefficient of 0.999. The relative standard deviation for 10 replicated injections was 2.3% at the 100 ppm sulfite level.

*Application and comparison*

Table I shows the results of sulfite determination in various kinds of white wine using this system. The results are compared with those obtained with the F-kit method proposed by Boehringer Mannheim GmbH. The determined values using the present system coincided relatively well with the F-kit method ones. This shows that the gelatin treatment is effective for white wine because of the latter's relatively low level of interfering substances. However, somewhat erratic values were obtained for sulfite in red wine. This means that the gelatin treatment is not sufficient for red wine in spite of its elimination efficiency of 94%. This is because red wine contains large amounts of polyphenol compounds, compared to the amount of sulfite. The elimination of interfering substances in red wine, including the exchange of the dialysis membrane with an other one, is under investigation.

### References


