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SIMULTANEOUS DETERMINATION OF SULFITE AND PHOSPHATE IN WINE BY MEANS OF IMMOBILIZED ENZYME REACTIONS AND AMPEROMETRIC DETECTION IN A FLOW-INJECTION SYSTEM

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Summary—A flow-injection system is proposed for the simultaneous determination of sulfite and phosphate in wine. A sulfite oxidase immobilized reactor and purine nucleoside phosphorylase–xanthine oxidase co-immobilized reactor are incorporated at fixed positions (parallel configuration) in the flow line, which is based on the splitting of the flow after sample injection and subsequent confluence. A poly(1,2-diaminobenzene)-coated platinum electrode is used as an amperometric detector to detect selectively hydrogen peroxide generated enzymatically in the enzyme reactors, without any interference from oxidizable species and proteins present in wine. Because each channel has a different residence time, two peaks are obtained. The first peak corresponds to sulfite and the second peak to phosphate. The peak current is linearly related to the concentrations of sulfite between 1×10^{-5} and $2 \times 10^{-3}M$ and phosphate between 2×10^{-5} and $5 \times 10^{-3}M$. The simultaneous determination of sulfite and phosphate in wine can be performed at a rate of 30 samples/hr with satisfactory precision (less than 1.2% RSD) and no pretreatment except for the sample dilution.

Amperometric flow-injection analysis (FIA) with immobilized enzyme reactors has become recognized as a selective, simple, accurate and rapid technique.[1–5] In such FIA systems, solid electrode-based detectors (*e.g.* platinum and glassy carbon electrodes) are commonly used for the detection of electroactive products (*e.g.* H_2O_2 and NADH) generated enzymatically in the enzyme reactor. However, these electrodes are often hampered by a gradual fouling of the electrode surface and also by a signal due to the electroactive interferences, especially in the analysis of the samples containing complex matrices such as serum, food *etc.*

Recently, many attempts have been made to offer higher selectivity and stability to amperometric detection by permselective coatings of electrode surface[6] (based on charge exclusion and size exclusion). A cellulose acetate coating[7] was first used to prevent adsorption of proteins on the electrode surface. Similarly, perfluorosulfonated Nafion[8–10] and polyester sulfonic acid[11] were used to prepare the polyanionic film on the electrode surface, which has ion-exchange properties that make it permeable to cations and impermeable to anions. These charged film-coated electrodes demonstrated a substantial improvement in differentiation of the primary amine (cationic) neurotransmitters from metabolites.[12,13]

On the other hand, electropolymerization has been also used as a valuable method for preparing a size-exclusion film on the electrode surface. Polyphenol-,[14] poly(1,2-diaminobenzene)-[15] and poly(*N*,*N*-dimethylaniline)-coated[16] electrodes were used to improve the selectivity of amperometric detection. If such polymer-coated electrodes are used as an amperometric detector in a flowing stream, the need for sample pretreatment may be reduced.

This paper describes the utility of the poly(1,2-diaminobenzene) film-coated platinum electrode in the amperometric detection of hydrogen peroxide generated enzymatically in the hydrogen peroxide-producing oxidase immobilized reactors. Furthermore, an attempt to apply this electrode to the simultaneous determination of sulfite and phosphate in wine is described, by using a flow system based on a

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parallel configuration of two enzyme immobilized reactors based on splitting of the flow after the sample injection and subsequent confluence of the streams.

The determination of sulfite and phosphate is especially important in the field of food and environmental analysis A variety of methods for determining sulfite and phosphate are reported: spectrophotometry,[17,18] ion chromatography[19,20] and an enzyme method[21,22] for the determination of sulfite and spectrophotometry,[23,24] ion chromatography,[25] ion selective electrode[26] and enzyme electrode[22,27] for the determination of phosphate. However, most of these methods usually involve some complicated and time consuming procedures. In contrast to these methods, the proposed amperometric FIA method is a simple and rapid technique and, furthermore, worked without any interferences from electroactive species and proteins present in wine.

EXPERIMENTAL

Reagents

Distilled water was used throughout. Purine nucleoside phosphorylase (EC 2.4.2.1, 14 U/mg, from bacterial), xanthine oxidase (EC 1.1.3.22, 17.4 U/ml, from buttermilk), and sulfite oxidase (EC 1.8.3.1, 89.6 U/ml, from chicken liver) were obtained from Sigma. Gluataraldehyde (20% solution), 1,2-diaminobenzene, sodium dihydrogenphosphate and sodium sulfite were purchased from Wako. They were used as received. All other chemicals were of analytical reagent grade. Phosphate and sulfite standard solutions (50 m*M* each) were prepared from sodium dihydrogen phosphate and sodium sulfite, respectively. The Tris–HCl buffer was prepared from 0.1*M* tris(hydroxymethyl)-aminomethane solution by adjusting to an appropriate pH value with 1*M* HCl.

Preparation of immobilized enzyme reactor

The method was similar to that described previously.[28] Controlled-pore glass (aminopropyl-CPG, pore size 585 Å, particle size 200–400 mesh), obtained from CPG Inc. (Fairfield, NJ, U.S.A.), was packed into two glass columns (3 mm i.d.; 27 and 18 mm long, respectively) furnished with small nylon nets (300 mesh) at the ends. Glutaraldehyde solution (4% v/v) in 0.1*M* sodium hydrogen carbonate was circulated for 2 hr to activate the glass beads. After washing with 0.1*M* buffer (pH 7.2), purine nucleoside phosphorylase (32 U) and xanthine oxidase (1.8 U) were co-loaded onto the 27 mm column by circulating enzyme/borate buffer (0.1*M*, pH 7.2) solution for 2 hr at room temperature. Sulfite oxidase (50 U) was loaded onto the 18 mm column by circulating enzyme/phosphate buffer (0.1*M*, pH 7.5) according to the same procedure. The excess of enzymes and the residual aldehyde groups on the CPG were removed by washing with glycine buffer (0.1*M*, pH 7.5) for 2 hr. The reactors were stored at *ca.* 5°C in 0.1*M* Tris–HCl buffer at pH 7.5 when not in use.

Construction of poly(1,2-diaminobenzene) film-coated platinum electrode

An Eicom (Kyoto, Japan) thin-layer electrochemical flow-cell was used for the surface modification of the electrode. The electrode assembly consisted of a platinum disk (3 mm in diameter) as a working electrode, a silver–silver chloride reference electrode, and a stainless steel tube as an auxiliary electrode. The poly(1,2-diaminobenzene) film-coated platinum electrode was prepared by a similar procedure as described by Sasso *et al.*[15] Prior to the coating, the surface of the platinum disk was polished with alumina particles (Fujimi Metapolish, no. 4), rinsed with distilled water, then washed with distilled water in an ultrasonic bath and allowed to air-dry. The electropolymerization to coat the same surface with the thin film of poly(1,2-diaminobenzene) was carried out by holding, for 30 min, the working electrode at 1.0 V *vs.* Ag/AgCl in a flowing stream (pumped at a flow rate of 0.3 ml/min) of a solution of 1,2-diaminobenzene (20 m*M*) in 0.2*M*, pH 5.2, sodium acetate buffer that was deaerated with high-purity grade nitrogen before use, because 1,2-diaminobenzene was easily air-oxidized. After the electropolymerization, the electrode was exhaustively washed with distilled water. The electrode was stored in distilled water when not in use.

Apparatus and procedure

A FIA system shown in Fig. 1a was used for optimization of parameters which influence the enzymatic behavior, by positioning the enzyme reactor as shown.

Another flow system (Fig. 1b) is based on splitting of the flow after the sample injection point, passing through two channels with different dimensions and subsequent confluence of the streams before reaching the poly(1,2-

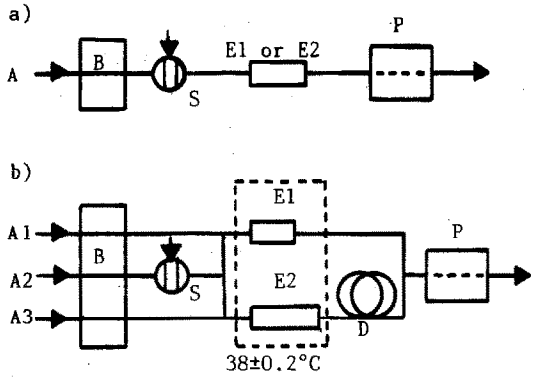

Fig. 1. (a) Single channel FIA manifold used for optimization of parameters. (b) FIA manifold used for the simultaneous determination of sulfite and phosphate. A, A1, A2 and A3: carrier solution; B: pump; S: injector; D: delay coil; E1: sulfite oxidase immobilized reactor; E2: purine nucleoside phosphorylase/xanthine oxidase co-immobilized reactor; P: poly(1,2-diaminobenzene)-coated platinum electrode.

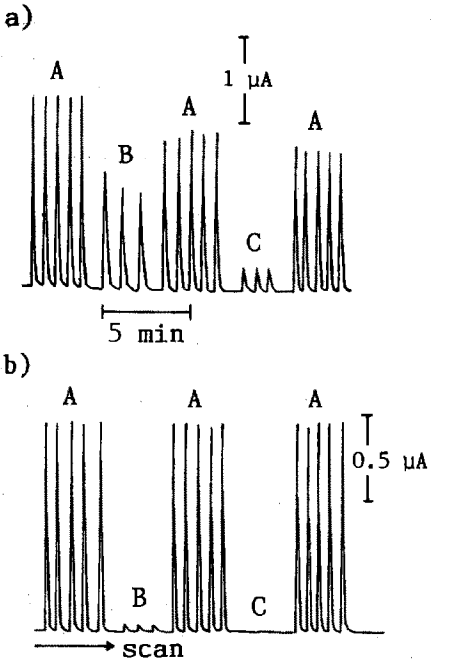

Fig. 2. Flow-injection current/time profiles of a bare platinum electrode (a) and poly(1,2-diaminobenzene)-coated platinum electrode (b) to the following: A, H_2O_2 (0.45 m*M*); B, commercial wine; C, 1:4 diluted wine. A 5-μl aliquot was injected into the single channel FIA system (Fig. 1a) without the immobilized enzyme reactor. The carrier solution (0.1*M* phosphate buffer, pH 7.5) was pumped at a flow rate of 1.0 ml/min.

diaminobenzene)-coated platinum electrode. This flow system was used for the simultaneous determination of sulfite and phosphate. Enzyme reactors were immersed in a water bath thermostatted at 38 ± 0.2°C. A constant potential (0.6 V *vs.* Ag/AgCl) was applied to the poly(1,2-diaminobenzene)-coated platinum electrode with a Yanagimoto VMD-101 potentiostat, and current was recorded on a Hitachi Model 056 strip-chart recorder. Carrier solution A1 (0.2*M* Tris–HCl buffer, pH 8.7, containing 0.1*M* NaCl), carrier solution A3 (0.2*M* Tris–HCl buffer, pH 7.5, containing 10 m*M* inosine and 0.1*M* NaCl), and distilled water (A2) were pumped at 0.5, 0.5, and 1.0, ml/min, respectively, with a Sanuki 4P3U-4036 pump. Sample solutions (10 μl) were injected with an injection valve (Rheodyne 7125) equipped with a 100 μl sample loop.

RESULTS AND DISCUSSION

Permselectivity of poly(1,2-diaminobenzene) film for hydrogen peroxide

A bare platinum electrode without surface modification gave a fairly large signal (Fig. 2a) owing to electroactive interferences (probably L-ascorbic acid, uric acid, cysteine, *etc.*) present in wine and further caused a decrease in sensitivity due to electrode fouling by surfactants such as proteins, when a wine sample (or 1:4 diluted) was injected repeatedly into the single channel flow-injection system (Fig. 1a) without the enzyme reactor. This means that the bare platinum electrode is not suited as an amperometric FIA detector of samples containing complex matrices such as wine.

On the other hand, the sensitivity of the poly(1,2-diaminobenzene)-coated platinum electrode to hydrogen peroxide was decreased to 56%, as compared with that of the bare electrode (Fig. 2). However, the signal current owing to the interferents decreased up to the negligible value and that owing to hydrogen peroxide remained constant even after repeated injections of wine sample (Fig. 2b). Also, this polymer-coated electrode effectively excluded the L-ascorbic acid, uric acid and L-cysteine below 2 m*M* and kept its desired properties for repetitive use over a period of three weeks. Such properties (selectivity and sensitivity for hydrogen peroxide) of the electrode were not much influenced by the electropolymerization conditions, although a variety of 0.1*M* phosphate buffers ranging in pH from 3 to 8 were used as the electrolyte solution and the electrolysis time was increased to 1 hr.

From these results, it is apparent that the poly(1,2-diaminobenzene)-coated platinum electrode prevents the oxidizable interferents and surfactants such as proteins, commonly

founds in wine, from reaching the electrode surface and so is very useful as an amperometric detector which responds selectively for hydrogen peroxide produced in the FIA system with hydrogen peroxide-producing oxidase immobilized reactor. Thus poly(1,2-diaminobenzene) film worked as a size-exclusion film to exclude effectively interferents in wine, in particular to allow the complete exclusion for 1:4 diluted wine sample (Fig. 2b).

Characterization of immobilized enzyme reactors

The reactions that occur in two immobilized enzyme reactors are as follows:

$$\text{Sulfite} + O_2 + H_2O \xrightarrow{\text{sulfite oxidase}} \text{sulfate} + H_2O_2 \quad (1)$$

$$\text{phosphate} + \text{inosine} \xrightarrow{\text{purine nucleoside phosphorylase}} \text{hypoxanthine} + \text{ribose-1-phosphate} \quad (2)$$

$$\text{hypoxanthine} + 2O_2 \xrightarrow{\text{xanthine oxidase}} \text{uric acid} + 2H_2O_2. \quad (3)$$

Sulfite is enzymatically oxidized to produce hydrogen peroxide in the sulfite oxidase immobilized reactor, whereas phosphate results in the production of uric acid and hydrogen peroxide as end-products by successive enzymatic reactions (equations 2 and 3) in the presence of inosine in the purine nucleoside phosphorylase–xanthine oxidase co-immobilized reactor. The hydrogen peroxide produced is amperometrically detected with the flow-through poly(1,2-diaminobenzene)-coated platinum electrode downstream, which enables the selective detection of hydrogen peroxide because of blocking access of uric acid to the electrode surface.

Experiments were carried out to establish the optimum conditions for both enzyme reactors. The variables affecting the performance of the enzyme reactors were optimized by using the single-channel FIA system shown in Fig. 1a. Table 1 shows the range over which each variable was studied and the optimal value found. Tris–HCl buffers at various pH values were tested as carrier solution. Maximum response for sulfite and phosphate was obtained at pH 8–9 and 7–8, respectively. Without inosine in the carrier solution, however, no response to phosphate was observed. As the inosine concentration was raised, the sensitivity to phosphate was increased, but the increase was slight at concentrations greater than 5 mM. A 5 mM concentration was selected as optimal. Also, 50 mM sodium chloride was added to the carrier solution to increase the ionic strength, because the sensitivity of the electrode to hydrogen peroxide was increase up to 50 mM concentration of sodium chloride. Therefore, 0.1M Tris–HCl buffer of pH 8.7 containing 50 mM NaCl and 0.1M Tris–HCl buffer of pH 7.5 containing 5 mM inosine and 50 mM NaCl were selected as the optimum carrier solutions, for the use of the sulfite oxidase immobilized and purine nucleoside phosphorylase–xanthine oxidase co-immobilized reactors, respectively. Also, the optimum carrier flow-rate was 1.0 ml/min

Table 1. Optimization of variables for use of enzyme reactors and their analytical characteristics

Column	Variable	Range studied	Enzyme reactor	
			SOD*	PNP/XO†
Reactor size (3 *mm* i.d.)	Length (*mm*)	12–27	18	27
Chemical variable	(carrier solution)			
	Buffer	—	Tris–HCl (0.1M)	Tris–HCl (0.1M)
	pH	6.0–9.5	8.7	7.5
	Inosine (*mM*)	0–10	—	5
	NaCl (*mM*)	0–100	50	50
Physical variable	Reactor temperature (°C)	18–48	room temp.	38 ± 0.2
	Flow rate (*ml/min*)	0.3–2.0	1.0	1.0
Characteristic	Enzymatic conversion efficiency (%)	—	100	100
	Linear calibration range (M)‡	10^{-6}–10^{-2}	5×10^{-6}–2×10^{-3}	5×10^{-6}–2×10^{-3}
	RSD (%)§		0.87	0.92

*Sulfite oxidase immobilized reactor.
†Purine nucleoside phosphorylase/xanthine oxidase co-immobilized reactor.
‡For 5 μl injections.
§At a concentration of $2 \times 10^{-4}M$ each ($N = 7$).

for each reactor. The temperature of the reactors had no effect on the signal of sulfite. However, the signal of phosphate was increased with increase of the reactor temperature, becoming almost constant about 38°C. The optimum length of reactor (3 mm i.d.) was 18 and 27 mm, respectively, for the measurement of sulfite and phosphate.

Under such optimum conditions, the conversion efficiency of sulfite and phosphate to hydrogen peroxide was *ca.* 100%; this was calculated by comparison of signal current of each species and that of hydrogen peroxide, because 1 and 2 mol of hydrogen peroxide should be produced for every mole of sulfite and phosphate, respectively, after complete enzymatic conversion. The linear calibration graph was obtained over 5×10^{-6}–$2 \times 10^{-3}M$ for each species of sulfite and phosphate (5 μl injections), with the linear correlation coefficients larger than 0.998. The relative standard deviation of signal currents for seven replicate injections was less than 1% for each species at a concentration of $2 \times 10^{-4}M$.

Simultaneous determination of sulfite and phosphate in wine

The flow system shown in Fig. 1b was used for the simultaneous determination of sulfite and phosphate in wine. The injected sample (10 μl) is split into two streams at the T-connector after the sample injection point, and the two portions pass through two channels with two different immobilized enzyme reactors, with subsequent confluence of the streams before reaching the poly(1,2-diaminobenzene)-coated platinum electrode. As examined at the previous section, the optimum pH values of carrier A1 (0.2*M* Tris–HCl buffer containing 0.1*M* NaCl) and carrier A3 (0.2*M* Tris–HCl buffer containing 10 m*M* inosine and 0.1*M* NaCl) were 8.7 and 7.5, respectively. When both carriers (A1 and A3) were pumped together at a flow rate of 0.5 ml/min with carrier A2 (distilled water pumped at 1.0 ml/min), each of sulfite and phosphate was quantitatively converted to hydrogen peroxide by the enzymatic reactions in each channel. Two peaks were obtained for injections of a mixture of sulfite and phosphate as shown in Fig. 3, because each channel has a different residence time. The first peak corresponded to sulfite and the second to phosphate.

The separation of the two peaks depended on the relative length of the channels; a delay PTFE coil, 2.0 m × 0.5 mm i.d., was needed to separate

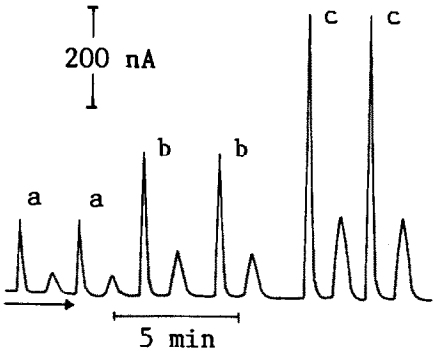

Fig. 3. Typical FIA signals obtained for simultaneous determination of sulfite and phosphate: (a) 0.25 m*M* each of sulfite and phosphate; (b) 0.5 m*M* each of sulfite and phosphate; (c) 1 m*M* each of sulfite and phosphate. Injection volume: 10 μl. The first (sharp) peak corresponds to sulfite and the second (broad) to phosphate.

the peaks completely. The calibration graphs for both species were run using standard solutions of sulfite and phosphate with concentrations between 1×10^{-6} and $1 \times 10^{-2}M$, which were injected in triplicate into the optimized simultaneous flow system. Linear relation between signal currents and each species was observed in the range of 1×10^{-5}–$2 \times 10^{-3}M$ (sulfite) and 2×10^{-5}–$5 \times 10^{-3}M$ (phosphate); the upper limit is probably restricted because of oxygen limitation in the carrier solution, while the lower limit is restricted because of fluctuation of the base-line. The slope, the y intercept, and the linear correlation coefficient were 608 nA/m*M*, 1.1 nA and 0.998 for sulfite, and 185 nA/m*M*, 0.8 nA and 0.996 for phosphate. The reproducibility of the measurements was tested by repeated injections ($N = 10$) of 10 μl aliquots of standard solution containing $4 \times 10^{-4}M$ each of sulfite and phosphate. The relative standard deviation were 0.81 and 1.2% for sulfite and phosphate, respectively. Up to 30 samples per hour could be analyzed under optimized flow conditions.

The selectivity of the method depends on that of the enzymatic reaction used and that of the electrode used as detector. Foreign species were added to the standard solution (containing 0.2 m*M* each of sulfite and phosphate) at higher concentrations (10 m*M*) and the signals obtained were compared with that from the same standard solution containing no foreign species; ions such as sulfate, nitrate, nitrite, chlorate, iodate, bromate, carbonate, pyrophosphate, thiosulfate, thiocyanate, acetate, lactate, tartrate, citrate, succinate, oxalate, and ammonium did not interfere at a mole ration of 50:1

(foreign ion to analyte). Also the co-immobilized purine nucleoside phosphorylase–xanthine oxidase and the immobilized sulfite oxidase were themselves highly selective for each of phosphate and sulfite. On the other hand, the poly(1,2-diaminobenzene) film-coated electrode functioned as a size-exclusion film to exclude the electroactive interferents and proteins, commonly found in wine. Actually the magnitude of the peak current due to the electroactive interferents in wine was *ca.* 0.5% of that 2 m*M* sulfite (normal concentration in wine). Consequently, the proposed FIA system offered high selectivity for the simultaneous determination of sulfite and phosphate. The ethanol content in the sample did not affect the signal currents of sulfite and phosphate at the concentrations below 5% (v/v). At higher concentrations, however, signal currents decreased slightly, probably because the activity of the immobilized enzymes decreased in the presence of higher concentration (>5%) of ethanol.

The proposed FIA system was applied to the simultaneous analysis of sulfite and phosphate in wine. The samples were diluted with distilled water by a factor of five and then their 10 μl aliquots were introduced into the flow system shown in Fig. 1b. The samples were also analyzed by spectrophotometric methods for sulfite[18] and phosphate[24] determinations. The analytical results by the proposed method were in good agreement with data obtained by the spectrophotometric method (Table 2). The correlation coefficient between the two methods were found to be 0.971 for sulfite and 0.998 for phosphate. The reproducibility of the results was tested by repeated injections ($N = 10$) and the relative standard deviations were less than 1.2% for all samples.

Two immobilized enzyme reactors were reasonably stable; even after repetitive use for 50 days, they retained the sufficient enzyme activities which could enable complete enzymatic conversion, but after that their enzyme activities decreased gradually. Also, the poly(1,2-diaminobenzene)-coated platinum electrode was used repeatedly to confirm the long term stability; even after repetitive use (about 10 wine samples/day) at room temperature for three weeks, it was stable enough to use, but after that lost its desired properties rapidly.

Table 2. Analytical results* for simultaneous determination of sulfite and phosphate in wine

	Present FIA method		Spectrophotometric method[18,24]	
	Sulfite	Phosphate	Sulfite	Phosphate
Sample	(*mM*)	(*mM*)	(*mM*)	(*mM*)
1	2.60	2.65	2.38	2.52
2	2.05	2.85	1.96	2.59
3	2.70	3.25	2.92	3.15
4	1.05	4.05	1.18	4.22
5	2.60	4.50	2.38	4.28
6	3.25	9.00	3.31	9.35
7	2.90	3.65	2.98	3.58

*Results are the average values of data obtained by five measurements.

CONCLUSIONS

An attempt to apply the poly(1,2-diaminobenzene)-coated platinum electrode to the detection of hydrogen peroxide generated enzymatically into the enzyme reactor afforded the highly selective and simultaneous determination of sulfite and phosphate in wine, by using a flow system based on splitting of the flow after the sample injection point and subsequent confluence of the streams. As the proposed FIA method was based on the combination of the substrate selectivity of the enzymatic reaction and permselectivity of the polymer-coated electrode, it may be applied to the rapid determination of sulfite and phosphate without any interferences. This method can be further applied to the determination of a variety of substrates in sample (such as serum, food, *etc*) containing complex matrixes, by using other hydrogen peroxide-producing oxidase immobilized reactors.

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