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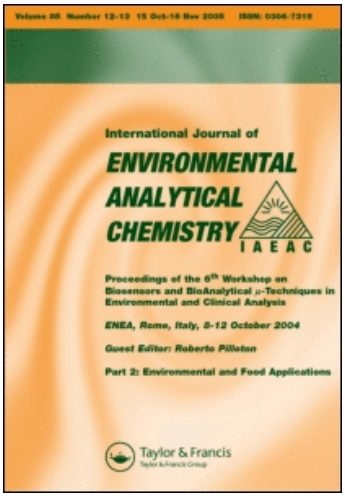

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Use of Enzymes for the Determination of Environmental Pollutants†

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This paper describes three enzyme-based analytical methods for sulfur dioxide, formaldehyde, and formic acid using sulfite oxidase, formaldehyde dehydrogenase, and formate dehydrogenase, respectively. In the determination of sulfur dioxide, air samples are collected in triethanolamine buffer solution and then reacted with sulfite oxidase/NADH/NADH peroxidase solution. The decrease in NADH, which is proportional to the concentration of sulfur dioxide, is determined spectrophotometrically at 340 nm or fluorometrically at λ_{ex} of 340 nm and λ_{em} of 467 nm. In the determination of formaldehyde, formaldehyde dehydrogenase catalyzed the reaction of formaldehyde with NAD^+ to form NADH which can be measured spectrophotometrically. For higher sensitivity and better detection limit, NADH is coupled to INT/diaphorase to produce formazan, a highly chromogenic compound. In the determination of formic acid, formate dehydrogenase is employed and NADH formed is measured spectrophotometrically. The NADH produced is also coupled to the diaphorase-catalyzed reduction of resazurin to form resorufin which can be measured fluorometrically.

KEY WORDS: Enzymatic analysis, sulfur dioxide determination, formaldehyde determination, formic acid determination.

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I. INTRODUCTION

During the past decade, enzymes have become increasingly useful as analytical reagents due to their specificity. Many enzymes are specific for a particular reaction of a particular substrate even in the presence of other isomers of that substrate or similar compounds. Enzymes are also capable of catalyzing the reaction of a substrate at very low concentrations. These facts, together with the advent of analytical instrumentation, encourage the use of enzymes for quantitation of various substrates, inhibitors, or activators. The growing analytical applications of enzymes have been reflected in extensive publications in recent years.[1,2] However, most of these applications are in clinical and bioanalytical chemistry. This paper presents the use of enzymes as analytical reagents for the determination of environmental pollutants. Three enzyme-based analytical methods for sulfur dioxide, formaldehyde, and formic acid using sulfite oxidase, formaldehyde dehydrogenase, and formate dehydrogenase, respectively, are described.

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Sulfur dioxide is probably one of the most important air pollutants. The widespread occurrence in industrial and power generation processes as well as the documented toxicity[3] of sulfur dioxide create a need for a simple, sensitive, and specific method for the detection of this pollutant. Spectrophotometric method, in which sulfur dioxide is collected in a solution of tetrachloromercurate and then reacted with pararosaniline and formaldehyde solution to form a colored product, is currently a recommended procedure.[4] However, this method is not free from interferences inherent in non-enzymatic reaction.

Determination of formaldehyde in air and in water is important. Formaldehyde is widely used in a large number of industrial applications such as urea formaldehyde foams, phenolic resins, particle board, plywood, textiles, as well as starting material for the manufacture of many other chemicals. The health effects and potential of carcinogenicity associated with formaldehyde exposure in rats[5-7] created great concern on the controlling and monitoring of exposure to this chemical, both in working and indoor environments. Several spectrophotometric,[8-10] chromatographic[11-15] and polarographic[16] methods have been developed for formaldehyde determination.

Urinary formic acid concentrations can be used as an indicator for the monitoring of formaldehyde exposure. Even though the levels of urinary formic acid have not yet been correlated well with the extent of formaldehyde exposure,[17] Einbrodt *et al.*[18] reported that the formic acid levels in urine increase by a factor of 3 to 7 in workers exposed to 0.93–1.19 ppm formaldehyde for 8 hours. The excretion rate, or total amount excreted, of formic acid in urine as well as the level of this chemical in blood, have also been considered as possible parameters for the biological monitoring of exposure to acetone[19] and methanol.[20,21]

II. DETERMINATION OF SULFUR DIOXIDE

In an aqueous solution, sulfur dioxide exists as sulfite and can be enzymatically oxidized to sulfate by sulfite oxidase (EC 1.8.3.1) or sulfite dehydrogenase (EC 1.8.2.1), or reduced to sulfide by sulfite reductase (EC 1.8.1.2). Sulfite oxidase, which was isolated from chicken liver, catalyzes the following reaction:[22]

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

The Michaelis constant (K_m) of this enzyme toward sulfite is 2.4×10^{-5} M in 0.2 M Tris buffer at pH 8.5 and 25°C.[23] Sulfite oxidase can be used for the quantification of sulfur dioxide in air by measuring the amount of H_2O_2 produced spectrophotometrically using peroxidase or the amount of O_2 consumed electrochemically using O_2 electrode. However, two problems arose and need to be solved. First, the H_2O_2 formed in the reaction 1 can react with the excess sulfite in the assay solution.[24] Second, sulfur dioxide is unstable in aqueous solution due to the oxidation of sulfite to sulfate by oxygen. Usually sulfur dioxide in the air is collected by aspirating a measured volume of air sample through a solution of tetrachloromercurate in which sulfur dioxide is stabilized as tetrachlorosulfitomercurate complex.[4] This procedure resists oxidation by oxygen or other strong oxidants such as ozone and oxides of nitrogen. We were unable to use tetrachloromercurate as a stabilizer in the determination of sulfur dioxide using sulfite oxidase because tetrachloromercurate inhibited the enzyme. These two problems affect the reproducibility, sensitivity, and detection limit of the method and

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therefore must be overcome. The first problem can be solved by employing the second enzyme, NADH peroxidase (EC 1.11.1.1), which catalyzes the following reaction:[22]

$$H_2O_2 + NADH + H^+ \xrightarrow{\text{NADH peroxidase}} 2H_2O + NAD^+. \quad (2)$$

This reaction serves two purposes. First, it effectively removes H_2O_2 under the assay conditions due to the high reaction rates of both sulfite oxidase and NADH peroxidase as compared to the chemical reaction between H_2O_2 and sulfite. The K_m of NADH peroxidase is 2.8×10^{-5} in 0.2 M Tris buffer at pH 6 and 25°C.[25] Furthermore, under the alkaline condition of the assay system, the reaction between H_2O_2 and sulfite is relatively slow. Second, reaction 2 provides a means for sulfite quantification. The decrease in NADH, which is proportional to the concentration of sulfite, is determined spectrophotometrically at 340 nm or fluorometrically at λ_{ex} of 340 nm and λ_{em} of 467 nm. The second problem can be solved by using triethanolamine (TEA) buffer as a reaction medium. Sulfur dioxide can be collected and stabilized in TEA buffer.

For the determination of sulfur dioxide, air samples are collected in TEA solution using an impinger. Sulfur dioxide concentrations are then determined by adding 100 μl of sample into a solution of NADH, NADH peroxidase (from *Streptococcus faecalis*), and TEA buffer. The solution is mixed thoroughly and the absorbance is monitored until a stable reading is obtained. Sulfite oxidase (from chicken liver, suspended in 3 M ammonium sulfate solution, pH 6.0) is then added and a decrease in absorbance at 340 nm is measured. The concentrations of these reagents in the assay solution are shown in Table I. For calibration, standard solutions are pre-

Table I Concentrations of reagents used in the determination of sulfur dioxide

Reagent	Concentration in assay solution
NADH	2.2×10^{-4} M
NADH peroxidase	47 U/L
Sulfite oxidase	40 U/L
TEA buffer (pH 8.0)	0.2 M

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pared from sodium sulfite. The actual concentration of sulfur dioxide in the standard solutions is determined by adding excess iodine and back titrating with sodium thiosulfate that has been standardized against potassium iodate.[4] The linearity of the calibration plot is from 0.7 to 10 μg/ml.

III. DETERMINATION OF FORMALDEHYDE

There are two types of formaldehyde dehydrogenase which can oxidize formaldehyde to formic acid: one requires glutathione as a cofactor and the other does not depend on glutathione. In 1974, Uotila and Koivusalo[26] reported that formaldehyde derived from methanol appears to be oxidized by the glutathione-dependent formaldehyde dehydrogenase (EC 1.2.1.1) in the cytosol. This enzyme was further described by Schutte *et al.*[27,28] In 1976, Cinti *et al.*[29] showed that formaldehyde derived from the microsomal N-demethylation reaction is oxidized by a non-glutathione-dependent formaldehyde dehydrogenase (EC 1.2.1.46) in mitochondria. In this study, we developed two analytical methods for the determination of formaldehyde using the latter enzyme. The principle of these methods is based on the oxidation of formaldehyde with NAD^+, catalyzed by the non-glutathione-dependent formaldehyde dehydrogenase isolated from *Pseudomonas putida*, to form formic acid and NADH:

$$\text{Formaldehyde} + NAD^+ + \xrightarrow{\text{Formaldehyde dehydrogenase}} \text{Formic acid} + NADH. \quad (3)$$

The equilibrium of this reaction is far in favor of the production of formic acid and formaldehyde can be determined quantitatively. In the enzymatic method 1, the NADH produced is measured spectrophotometrically at 340 nm. In the method 2, a second enzyme, diaphorase, is used to catalyze the reaction of NADH with the oxidized form of 2(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to produce formazan, a highly chromogenic compound:

$$NADH + INT \xrightarrow{\text{Diaphorase}} NAD^+ + \text{Formazan}. \quad (4)$$

Formazan is measured at 500 nm.

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For the determination of formaldehyde using method 1, a 500 μl of formaldehyde sample or standard is added to the solution of NAD^+ in phosphate buffer. The solution is mixed thoroughly and the absorbance is monitored until a stable baseline is obtained. After the zero reading is adjusted, formaldehyde dehydrogenase is added and the increase in absorbance versus time is monitored on a recorder. The final concentrations of reagents in the assay solution are shown in Table II. Formaldehyde can be determined in the linear range from 0.3 to 8 μg/ml. For method 2, glycine buffer (pH 9.0) is used. Formaldehyde standards or samples are added to a solution of NAD^+, diaphorase, and INT. The reaction is initiated by adding formaldehyde dehydrogenase and the absorbance of the formazan formed is monitored on a recorder. Concentrations of reagents used in this method are also shown in Table II. Formaldehyde can be determined in the linear range from 0.05 to 3 μg/ml.

These two enzymatic methods are simple, sensitive, and selective for formaldehyde. The interferences, reproducibility, correlation, as well as optimal conditions were investigated and reported elsewhere.[30] Cyanide, sulfide, and some aldehydes (acetaldehyde, propionaldehyde, and glyoxal) interfere only at high concentrations. At low concentrations, no interferences were observed with these compounds. These methods will be very useful not only for the analysis of formaldehyde in air but also in water and biological samples.

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Table II Concentrations of reagents used in the determination of formaldehyde

Reagent	Concentrations in the assay solution	
	Method 1	Method 2
β-NAD^+	0.77 mg/ml	0.14 mg/ml
Formaldehyde dehydrogenase	0.77 U/ml	0.67 U/ml
Diaphorase	—	1.80 U/ml
INT	—	0.16 mg/ml
Phosphate buffer (pH 8.0)	0.09 M	—
Glycine buffer (pH 9.0)	—	0.05 M

IV. DETERMINATION OF FORMIC ACID

Two enzymatic methods were developed for formic acid determination using formate dehydrogenase (EC 1.2.1.2). The principle of these methods is based on the oxidation of formic acid by NAD^+ in the presence of formate dehydrogenase.

$$HCOOH + NAD^+ \xrightarrow{\text{Formate dehydrogenase}} CO_2 + NADH + H^+. \quad (5)$$

The amount of NADH formed, which is proportional to formic acid in the assay solution, is measured spectrophotometrically at 340 nm.[31] For higher sensitivity and better detection limit, a second enzymatic method was also developed. The NADH produced is coupled to the diaphorase-catalyzed reduction of resazurin, a non-fluorescence compound, to resorufin, a highly fluorogenic compound:

$$NADH + \text{Resazurin} \xrightarrow{\text{Diaphorase}} NAD^+ + \text{Resorufin}. \quad (6)$$

Resorufin is measured at λ_{ex} of 565 nm and λ_{em} at 590 nm.[32-34] INT can also be used to react with NADH to form formazan, a highly chromogenic compound, as shown in reaction 4.

For the assay of formic acid using the first method, phosphate buffer (pH 7.5), NAD^+, and formate sample or standard are mixed thoroughly and the absorbance is measured until a stable baseline is obtained. The reaction is then initiated by adding formate dehydrogenase and the increase in absorbance is recorded. The concentrations of reagents used in this method are shown in Table III.

Table III Concentrations of reagents used in the determination of formic acid

Reagent	Concentration in the assay solution	
	Method 1	Method 2
β-NAD^+	5 mg/ml	0.66 mg/ml
Formate dehydrogenase	0.33 U/ml	2.5×10^{-3} U/ml
Diaphorase	—	0.4 U/ml
Resazurin	—	0.01 mg/ml
Phosphate buffer (pH 7.5)	0.05 M	2.0 mM
Phosphate buffer (pH 6.0)	—	0.1 M

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Urinary formic acid in the concentrations that ranged from 0.5–100 μg/ml can be determined with this method. Formate dehydrogenase is specific for formic acid. Acetic acid, ascorbic acid, propionic acid, and oxalic acid do not interfere at concentrations up to 100 μg/ml. Formaldehyde may reduce the rate of the reaction; however, it does not affect the determination of formic acid in urine up to 50 μg/ml.

For the determination of formic acid using the second method the reaction is carried out by two steps due to the different optimal pH conditions of formate dehydrogenase and diaphorase. In the first step, formate standards or samples are added to the solution of NAD^+ and formate dehydrogenase in phosphate buffer (pH 7.5). The solution is incubated for 15 minutes at 37°C. In the second step, phosphate buffer (pH 6.0), diaphorase, and resazurin are added to the solution. The contents are mixed and incubated for another 5 minutes at 37°C. The concentrations of reagents used in this method are also shown in Table III. The fluorescence is then measured. The working range of the calibration curve is from 0.030 to 0.42 μg of formate/ml solution. Formic acid in urine or blood samples in the concentration range of 2–25 μg/ml can be determined with this method.

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