

Accounts

Versatility of the Titanium(Ⅳ)–Porphyrin Reagent for Determining Hydrogen Peroxide

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Hydrogen peroxide has been an important analyte in many fields for many years. The Ti–TPyP reagent, i.e., an acidic aqueous solution of oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(Ⅳ) complex, was developed as a highly sensitive spectrophotometric reagent for determining traces of hydrogen peroxide. Following the addition of hydrogen peroxide to the reagent, the absorbance at 432 nm decreased and a new peak appeared at 450 nm (the Soret band) accompanied by the consumption of the complex and the formation of its monoperoxo complex, respectively. The degrees of the absorbance changes were found to be proportional to the hydrogen peroxide concentration with the apparent molar absorptivities of 1.9×10^5 (432 nm) and 1.1×10^5 (450 nm) $M^{-1}\,cm^{-1}$ ($1\ M = 1\ mol\,dm^{-3}$). Both values are much larger than those obtained by the conventional analysis methods. Based on these facts, the determination of hydrogen peroxide was made by a batch method and a flow injection analysis (FIA) method with the detection limits of 25 pmol and 0.5 pmol per test, respectively. In this account, the Ti–TPyP reagent is assessed for determining hydrogen peroxide in rainwater and in the atmosphere, and for determining several components in foods and biofluids mediated by appropriate oxidase enzymes, to demonstrate its potential for a broad range of applications.

In recent years, needs have been increasing among the analytical chemists for ways to determine traces of hydrogen peroxide. Hydrogen peroxide in the atmosphere and in environmental water has become of interest as the terminal product of hydroperoxyl radicals in photochemical reactions. Hydrogen peroxide functions as an oxidant in conversions of sulfur oxides to sulfuric acid, and also nitrogen oxides to nitric acid, in the atmosphere and rainwater, and such conversions sometimes lead to undesirable effects for biological systems.[1–5]

Because of such strongly oxidative functions, hydrogen peroxide is often used as a preservative or a bleaching agent in food manufacturing. However, since it was pointed out in 1980 that hydrogen peroxide appeared to exhibit carcinogenic activity, the amount of hydrogen peroxide allowed to remain in final food products has been officially regulated to be effectively nil.

The determination of hydrogen peroxide is regarded as significant in clinical assays, because a variety of components of physiological significance, such as glucose, uric acid, or cholesterol, are quantitatively oxidized with the corresponding oxidase enzymes, and the resulting hydrogen peroxide should be determined in high sensitivity.

A number of methods have been developed to determine hydrogen peroxide. However, most of these methods are based on the oxidation reactions of fluorogens or chromogens with hydrogen peroxide using peroxidase.[6–8] Because of the low selectivity of peroxidase to hydrogen donors (fluorogens or chromogens),[9] these methods are therefore liable to be affected by the presence of other oxidative and reducible substances concurrently present in the samples.

To avoid the interferences from such oxidative and reducible substances, scientists need a new sensitive, simple and rapid assay method for hydrogen peroxide with high selectivity. We have sought new ways to approach the effective color developing systems, not based on the redox reactions but on the formation of a metal complex containing both hydrogen peroxide and a dye substrate as ligands. Among the complexes examined, titanium(Ⅳ) complexes with hydrogen peroxide and porphyrin derivatives were found to be fitted to this purpose, owing to the strong affinity of titanium(Ⅳ) for hydrogen peroxide and a large light absorptivity of porphyrin at about 400–450 nm (the Soret band).

This article reviews the characteristics of the Ti–TPyP reagent, i.e., an acidic aqueous solution of oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(Ⅳ) complex, for determining traces of hydrogen peroxide, and the assessment procedures of the reagent in environmental, food and clinical analysis in order to demonstrate the versatility of the reagent for a broad range of applications.

Steps up to the Goal "Ti–TPyP Reagent"

Hydrogen peroxide has been noted as an important analyte in many fields. A large number of methods have been developed so far for the determination of hydrogen peroxide.

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Among them, luminescent and colorimetric methods incorporated with peroxidase mediated oxidation of fluorogens or chromogens to form hydrogen peroxide are commonly used as a routine manner in clinical assays.[6–8] For example, colorimetric methods are combined with the peroxidase mediated oxidation of a colorless chromogen substrate, such as 4-aminoantipyrine–phenol system, by hydrogen peroxide to form a colored product, such as quinoneimine dye. Although these methods provide high sensitivity, the obtained results are susceptible to the effects of concurrently present reducible substances, such as ascorbic acid and uric acid, in biological samples, since the methods are based on the redox reaction mechanisms. Such reducible substances also participate in the peroxidase mediated oxidation together with the chromogen (or fluorogen), leading to a negative error in the analytical results.

Since 1980, to prevent such errors arising from the reducible substances, we have investigated new color developing systems not based on the redox reactions but on the formation of complexes in the ternary systems of M^{m+}–Y–H_2O_2, where M^{m+} and Y denote a metal ion and a dye substrate, respectively.

Hydrogen peroxide is known to react with Ti(Ⅳ) to form yellow peroxotitanium(Ⅳ) complex in a strong acid solution, and the reaction has been used to determine hydrogen peroxide. However, because of its weak absorptivity (molar absorptivity: $\varepsilon = 6.40 \times 10^2$ $M^{-1}\,cm^{-1}$ at 415 nm),[10] the method is too insensitive for practical uses. In our earlier work, coordinative binding of a dye substrate (Y) to the binary Ti(Ⅳ)–H_2O_2 complex to form the ternary Ti(Ⅳ)–Y–H_2O_2 complex seemed to be likely to have much greater absorbance. To find the most favorable Y substrate, several compounds of phthalein complexon and pyridylazo groups were examined. Among the compounds investigated, 4-(2-pyridylazo)resorcinol (PAR) and 2-[(5-bromo-2-pyridyl)azo]-5-[*N*-propyl-*N*′-(3-sulfopropyl)hydrazino]phenol (PAPS) were found to be especially fitted to this purpose. The Ti–PAR[11] and Ti–PAPS[12] reagents thus developed were found to be particularly useful for determining hydrogen peroxide with high sensitivity and selectivity. Both reagents react with hydrogen peroxide to form stable red-purple complexes: Ti–PAR–H_2O_2 and Ti–PAPS–H_2O_2. Their apparent molar absorptivities for hydrogen peroxide were of the order of 10^4 $M^{-1}\,cm^{-1}$, and the absorbance values were stable and not susceptible to the reducible substances.[11,12] Characteristics of the reagents are listed in Table 1. For the Ti–PAR–H_2O_2 complex, the absorption at 508 nm is considered to be attributable to the conjugated double bond system of PAR with a resonance structure as shown in Fig. 1. The structure of the complex might be stabilized by bridging of the pyridine N atom and the Ti through hydrogen

Table 1. Characteristics of the Ti–PAR and Ti–PAPS Reagents for Determining Hydrogen Peroxide

Reagent	Ti–Y–H_2O_2 complex		Detection limit	
	λ_{max}	ε	pmol/assay	
	/nm	/$M^{-1}\,cm^{-1}$	Batch	FIA
Ti–PAR	508	3.6×10^4	600	4
Ti–PAPS	539	5.7×10^4	400	3

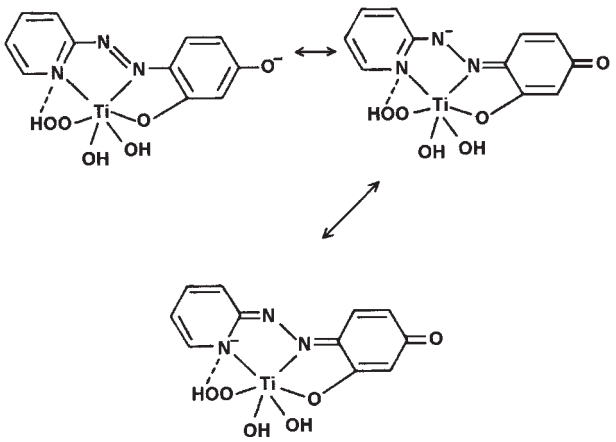

Fig. 1. Resonance structure of the Ti–PAR–H_2O_2 complex.[11]

bonding involving the H_2O_2.[11]

The reagents were actually applied to the determination of hydrogen peroxide remained in foods,[13,14] hydrogen peroxide in rainwater,[15,16] and to the determination of several components in human serum and urine mediated by the corresponding oxidase enzymes.[17–21]

Further, we had advanced our studies with the objective of attaining a more intensely absorbing Ti(Ⅳ) complex of $\varepsilon = 10^5$ $M^{-1}\,cm^{-1}$. Concurrently, certain water-soluble porphyrins were developed and became of interest among the analytical chemists as highly sensitive spectrophotometric reagents for various metal ions and other analytes.[22–28] Our objective was accomplished by introducing a water-soluble porphyrin derivative as the Y substrate to the Ti(Ⅳ)–Y complex. Oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(Ⅳ) complex, TiO(tpyp),[10] (see Fig. 2) was found to be particularly suited for our purpose, owing to its greater absorptivity at around 400–450 nm (the Soret band). Thus, an acidic aqueous solution of this complex, termed the Ti–TPyP reagent,[29] was proved to be a very sensitive reagent for hydrogen peroxide assay, based on the large molar absorptivity of the Ti–TPyP–H_2O_2 complex, ca. 10^5 $M^{-1}\,cm^{-1}$. Afterwards the Ti–TPyP reagent has been successfully used not only for the determination of hydrogen peroxide in environmental water,[29,30] but also for the determination of oxalate,[31] sulfite,[32] sugars,[33] fatty acids[34] in foods, and glucose,[35] uric acid[36] in human blood

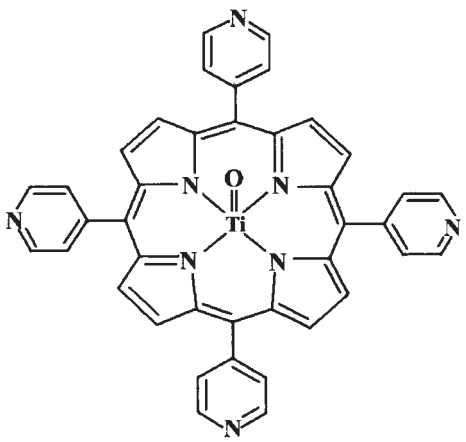

Fig. 2. TiO(tpyp) complex.[10]

and urine, generally mediated by the corresponding oxidase enzymes.

Characteristics of the Ti–TPyP Reagent for Determining Hydrogen Peroxide

1. Preparation of the Ti–TPyP Reagent. The TiO(tpyp) complex, synthesized according to the procedure of Inamo et al.,[10] was obtained from Tokyo Kasei Industries Co. The Ti–TPyP reagent was prepared by dissolving appropriate amounts of the TiO(tpyp) complex in an aqueous perchloric or sulfuric acid solution.[29–36] The concentrations of the reagent and acid were adjusted so as to optimize the reaction conditions in the final reaction mixture, as described below. For example, to prepare the Ti–TPyP reagent (3×10^{-5} M) containing 1 M sulfuric acid in the cases of flow injection analysis (FIA),[31–34] 10.0 mg of TiO(tpyp) was dissolved in 2 mL of 5 M hydrochloric acid, followed by adding 1 M sulfuric acid up to a total volume of 500 mL.

2. Absorption Spectra. The absorption spectrum of the Ti–TPyP reagent obtained in 0.5 M perchloric acid is shown in Fig. 3 as curve A.[29] The reagent exhibits a sharp peak at 432 nm, suggesting the presence of only the $TiO(tpypH_4)^{4+}$ complex as noted by Inamo et al., i.e., the four pyridyl groups of the porphyrin in the TiO(tpyp) complex are protonated in a perchloric acid solution.[10] Following the addition of hydrogen peroxide to the reagent, the absorption peak at 432 nm decreased significantly in proportion to the concentration of added hydrogen peroxide, this being the consumption of $TiO(tpypH_4)^{4+}$ accompanied by the appearance of a new peak at 450 nm[29] (Fig. 3, curve B). This new peak was assigned to peroxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(Ⅳ) complex, $Ti(O_2)(tpypH_4)^{4+}$.[10] Its absorbance was found to be proportional to the hydrogen peroxide concentration.[29] The formation of monoperoxo complex between $TiO(tpypH_4)^{4+}$ and H_2O_2 was confirmed by the molar ratio method.[29] Therefore, the reaction of the complex formation can be expressed as given in Fig. 4.

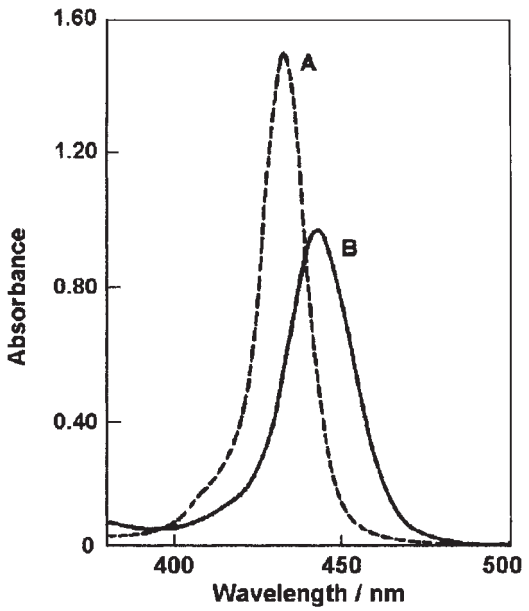

Fig. 3. Absorption spectra of A, the Ti–TPyP reagent; and B, $TiO_2(tpypH_4)^{4+}$ complex. [$HClO_4$]: 0.5 M; [Ti–TPyP]: 5×10^{-6} M; [H_2O_2]: 1×10^{-5} M.[29]

The equilibrium constant of this reaction was $(4.0 \pm 0.4) \times 10^6$ M at 25 °C,[10] which should be sufficient for determining hydrogen peroxide. According to the X-ray analysis of (5,10,15,20-tetraphenylporphynato)peroxotitanium(Ⅳ) by Guilard et al.,[37] titanium(Ⅳ) is favorably located at the center of the porphyrin plane, slightly atop the plane. In analogy with their study, the present monoperoxo complex is estimated to have a similar structure with a strain-free configuration, resulting in a very stable molecular structure.

In Fig. 3, the degree of the absorbance decrease at 432 nm per 1 M hydrogen peroxide (ΔA_{432}) was 1.9×10^5 M^{-1} cm^{-1}, while the apparent molar absorptivity for hydrogen peroxide at 450 nm (ε_{450}) was 1.1×10^5 M^{-1} cm^{-1}. Thus, both values are available for the determination of hydrogen peroxide.[29] When the determination is made by a batch method, use of the absorption decrease at 432 nm is recommended, because the larger value of ΔA_{432} makes it possible to determine the trace amounts of hydrogen peroxide with greater sensitivity. However, in the FIA of hydrogen peroxide, use of the absorbance at 450 nm is far preferable to keep the base-line lower, even the value of ε_{450} is somewhat smaller than that of ΔA_{432}. Characteristics of the Ti–TPyP reagent are summarized in Table 2.

3. Optimization of Reaction Conditions. The formation of the peroxo complex is relatively slow at room temperature. Addition of strong acids such as hydrochloric, sulfuric, nitric and perchloric acids was found to accelerate the formation of the complex. Among the acids examined, perchloric acid had the highest effect.[29] For example, in the presence of over 1.6 M perchloric acid, the reaction of hydrogen peroxide with

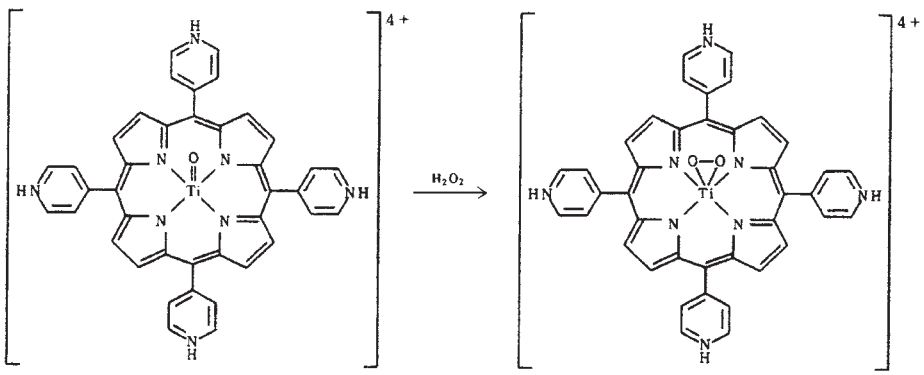

Fig. 4. Reaction of the Ti–TPyP reagent with H_2O_2 to form the monoperoxo complex, $TiO_2(tpypH_4)^{4+}$.

Table 2. Characteristics of the Ti–TPyP Reagent for Determining Hydrogen Peroxide

Notation	λ_{obs} /nm	ε /M^{-1} cm^{-1}	Detection limit (pmol/assay)	
			Batch	FIA
ΔA_{432}	432	1.9×10^5	25	—
ε_{450}	450	1.1×10^5	—	0.5

the Ti–TPyP reagent was equilibrated within 5 min. The highest sensitivity is attained at high perchloric acid concentrations; however, dealing with a large acid concentration leads to problems in the maintenance of an apparatus. Therefore, we adjusted the perchloric acid concentration in the Ti–TPyP reagent so as to be 1.6 M in the final reaction mixture.[29,30,35] Furthermore, use of perchloric acid causes a precipitate arising from proteins contained in food and biological samples. Such interference can be avoided by using sulfuric acid instead of perchloric acid. In such cases, the acid concentration in the reagent was adjusted so as to be 1 M in the final reaction mixture.[31–33,36]

In the batch method, the Ti–TPyP reagent of 5×10^{-5} M was used because it was found to be optimum for the peroxo complex formation. However, in the cases of FIA, we chose the Ti–TPyP reagent of 3×10^{-5} M to lower the base line of the flow signal.

Under these conditions, the absorbance values at 432 and 450 nm remained virtually unchanged for 2 h at room temperature. The Ti–TPyP reagent can be kept for 20 months in a refrigerator without any detectable changes in its absorbance.[29]

4. Effects of Foreign Substances.[29,30,34] To confirm the reliability of the analysis using the Ti–TPyP reagent, effects of some foreign substances concurrently present in real samples (such as environmental water, foods, biofluids) on the FIA signal of hydrogen peroxide were examined. As seen in Table 3, inorganic ions such as Na^+, K^+, Ca^{2+}, Ba^{2+}, Cl^-, Br^-, NO_3^- and SO_4^{2-} did not affect the analytical results much, even up to a concentration 5000-fold that of hydrogen peroxide (1×10^{-6} M). The presence of Ni^{2+}, Co^{2+} or Cu^{2+} up to a concentration 1000-fold gave no appreciable effects, whereas Fe^{3+} at more than 100 times the hydrogen peroxide concentration led to a negative error. Fe^{3+} is liable to catalyze the decomposition of hydrogen peroxide. However, such an effect might be practically negligible, because the Fe^{3+} content of rainwater is of the order of 10^{-8} M, and in biological samples, metal cations are mostly present not as free ions but in protein-binding states. In any event, the effects of metal cations can be easily removed, if necessary, by passing a test solution through a precolumn containing a cation-exchange resin prior to the measurements.

Various organic acids (except ascorbic acid) had no significant effects on the assay (Table 4). The effect of ascorbic acid was minor when its concentration was less than one-tenth part of hydrogen peroxide. On the other hand, the presence of ascorbic acid over one tenth parts caused an appreciable increase in the absorbance at 450 nm, leading to a positive error. This effect is due to the undesirable production of hydrogen peroxide through the catalytic oxidation of ascorbate by an enzyme-like effect of the Ti–TPyP reagent. Further discussion on this subject is described below.

Table 3. Effects of Inorganic Substances on the Determination of Hydrogen Peroxide Using the Ti–TPyP Reagent[30]

Substance added	Concentration /M	H_2O_2 found/%
NaCl	5×10^{-3}	101.4
KCl	1×10^{-2}	100.5
$CaCl_2$	5×10^{-3}	100.6
$MgCl_2$	1×10^{-2}	102.0
$BaCl_2$	5×10^{-3}	100.8
NH_4Cl	2×10^{-2}	101.6
$FeCl_3$	1×10^{-4}	97.6
$CuCl_2$	1×10^{-3}	99.4
$NiCl_2$	1×10^{-3}	100.8
$MnCl_2$	1×10^{-2}	102.4
$CoCl_2$	1×10^{-3}	99.2
NaBr	1×10^{-2}	102.6
$NaNO_3$	2×10^{-2}	101.2
NaN_3	5×10^{-2}	100.2
Na_2SO_4	1×10^{-2}	100.8
NaH_2PO_4	5×10^{-3}	100.2
H_3BO_3	5×10^{-3}	101.5

$[H_2O_2]$ added: 1.00×10^{-6} M.

Table 4. Effects of Organic Substances on the Determination of Hydrogen Peroxide

Substance added	Concentration /M	H_2O_2 found/%
Glycine	1×10^{-3}	99.5
Alanine	1×10^{-3}	98.5
Valine	1×10^{-3}	98.9
Leucine	1×10^{-3}	101.5
Isoleucine	1×10^{-3}	100.0
Serine	1×10^{-3}	99.7
Threonine	1×10^{-3}	101.6
Phenylalanine	1×10^{-3}	102.3
Tyrosine	1×10^{-4}	100.2
Tryptophan	1×10^{-5}	101.5
Aspartic acid	1×10^{-3}	97.6
Asparagine	1×10^{-3}	100.0
Glutamic acid	1×10^{-3}	99.7
Glutamine	1×10^{-3}	99.2
Lysine	1×10^{-4}	99.7
Histidine	1×10^{-3}	97.5
Cysteine	1×10^{-3}	102.7
Methionine	1×10^{-3}	97.8
Proline	1×10^{-3}	100.7
Ascorbic acid	1×10^{-8}	100.5
Albumin	0.5 mg/dL	101.3
Hemoglobin	0.05 mg/dL	98.1

$[H_2O_2]$ added: 1.00×10^{-6} M.

In practice, interference from ascorbic acid can be simply removed by passing a test solution through a precolumn containing immobilized ascorbate oxidase before the measurements. The Ti–TPyP reagent is thus shown to be effective for the selective determination of hydrogen peroxide.

Analytical Applications

1. Determination of Hydrogen Peroxide in Water. A procedure for determining hydrogen peroxide in water by a batchwise operation was as follows.[29] To a 250 μL water sample, 250 μL of perchloric acid (4.8 M) and 250 μL of the Ti–TPyP reagent were added, and the mixture was allowed to stand for 5 min at room temperature. The solution was diluted with water to 2.5 mL and this served as a test solution. A blank solution was prepared in a similar manner, using distilled water instead of the sample water. The absorbances of the test and the blank solutions were measured at 432 nm (denoted as A_S and A_B, respectively). The absorbance decrease (ΔA_{432}) was obtained by, $\Delta A_{432} = A_B - A_S$, from which the hydrogen peroxide content was determined.

The ΔA_{432} value was linear against the hydrogen peroxide concentration with the equation: $y = 1.9 \times 10^5 x + 0.0097$ (y and x being the ΔA_{432} and the molar concentration of hydrogen peroxide, respectively). The correlation coefficient was 0.999 over the range from 1.0×10^{-8} to 2.8×10^{-6} M (from 25 pmol to 7.0 nmol per test). Detection limit was 1.0×10^{-8} M (25 pmol per test). The relative standard deviation (RSD) was 1.2% at 1.0×10^{-6} M (2.5 nmol per test).

The method was readily applied to determine hydrogen peroxide in well water, tap water, ion-exchanged water and water treated by the NANO pure Ⅱ system (Barnstead), and the results are shown in Table 5. Hydrogen peroxide in ppb levels was determined for ten samples within 1 h.[29]

Since hydrogen peroxide in the atmosphere is known as one of the pollutants that cause acid rain, a more sensitive, rapid and simpler analysis method of hydrogen peroxide is highly desirable for checking hydrogen peroxide in rainwater. A FIA method using the Ti–TPyP reagent was thus assessed as an effective means to fill this demand.[30]

As shown in Fig. 5, a flow injection manifold comprises a two-channel system. CS was distilled water that served as a carrier and RS was the Ti–TPyP reagent (30 μM containing 1.6 M $HClO_4$), and both of them were made to flow at the flow rate of 0.4 mL min^{-1}. The flow lines were made of polytetrafluoroethylene tubing (0.5 mm i.d.). A 100 μL aliquot of water sample was directly injected into the carrier stream through the sample injector. Hydrogen peroxide contained in the water sample was allowed to merge with the reagent stream to form the peroxo complex in the mixing coil (15 m long, 0.5 mm i.d.). The peroxo complex was monitored at 450 nm by a spectrophotometer and its presence was recorded as the peak height of the flow signal. Typical flow signals obtained with the standard hydrogen peroxide are shown in Fig. 6. The response was linear against the hydrogen peroxide concentration ranging from 1×10^{-8} to 1×10^{-5} M (1.0 to 1000 pmol per test, $r = 0.999$) and the detection limit was 5×10^{-9} M (0.5 pmol per test, S/N = 2). The results were accurate with the RSD of 0.97% for the injection of 1×10^{-6} M of hydrogen peroxide ($n = 10$). The FIA operation conditions thus permitted 30 samples per hour to be processed. Because the procedure is simple, without any sample pretreatment prior to the injection into the FIA system, the present method enabled rapid analysis of hydrogen peroxide in water.

The detection limit obtained by FIA is considerably lower than that obtained by the batch method. In the batch method, the test solution was made by allowing the reaction of hydrogen peroxide with the Ti–TPyP reagent to proceed under highly acidic condition (1.6 M $HClO_4$), followed by diluting the reaction mixture up to 10-fold with water (cf., Ref. 29). Contrary to this, in the FIA method, hydrogen peroxide in the test solution was detected as the peroxo complex under a less di-

Table 5. Determination of Hydrogen Peroxide Content in Water Samples[29]

Sample source	H_2O_2 content /M	Recovery* /%
Well water	4.74×10^{-7}	90.6
Tap water	3.16×10^{-7}	84.0
Ion exchanged water	1.05×10^{-7}	96.9
NANOpureⅡ treated water	2.63×10^{-7}	99.2
Rainwater sample No. 1	2.74×10^{-6}	97.2
Rainwater sample No. 2	9.16×10^{-6}	94.9
Rainwater sample No. 3	1.62×10^{-5}	106.5
Rainwater sample No. 4	2.91×10^{-5}	104.4
Rainwater sample No. 5	3.34×10^{-5}	104.2

* $[H_2O_2]$ added: 3.00×10^{-7} M.

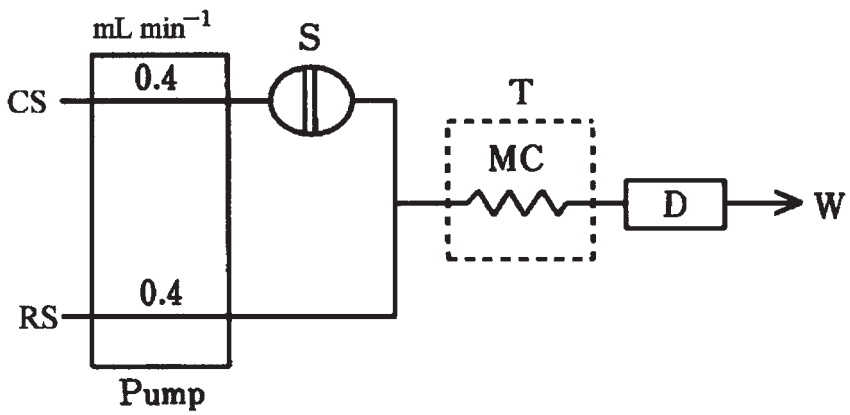

Fig. 5. Schematic diagram of the FIA system for the determination of hydrogen peroxide. CS: carrier stream (water); RS: Ti–TPyP reagent (3×10^{-5} M in 1.6 M $HClO_4$); S: 6-ways sampling valve (100 μL); T: thermostat bath (75 °C); MC: mixing coil (0.5 mm i.d., 15 m long); D: spectrophotometer (450 nm).[30]

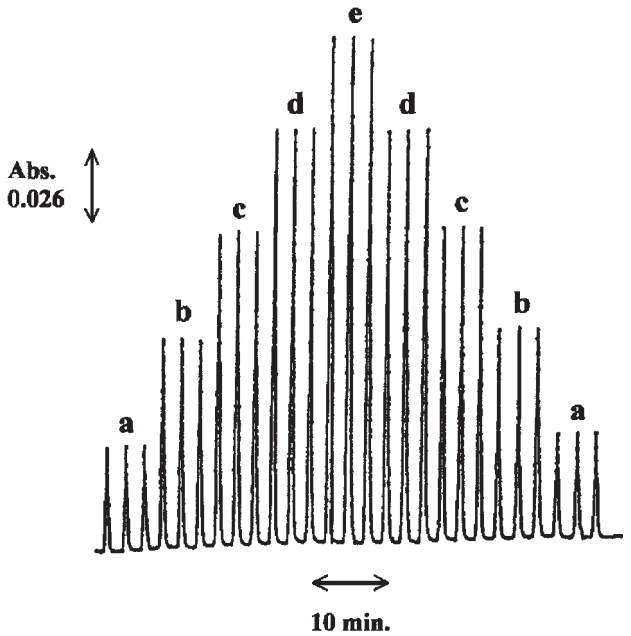

Fig. 6. Flow signals of the standard hydrogen peroxide. Amounts of hydrogen peroxide: a, 200; b, 400; c, 600; d, 800; e, 1000 pmol/100 μL injection.

Table 6. Hydrogen Peroxide Content in Rainwater Collected in Tokyo Area and the Recovery Values of Added Hydrogen Peroxide[30]

Sample No.	H_2O_2 content /M	Recovery* /%
1	7.0×10^{-7}	101.9
2	2.1×10^{-6}	99.4
3	4.3×10^{-5}	101.9
4	9.0×10^{-6}	101.5
5	1.2×10^{-5}	99.1
6	3.7×10^{-6}	101.7
7	2.4×10^{-6}	99.2
8	6.5×10^{-6}	101.6

* $[H_2O_2]$ added: 1.00×10^{-6} M.

luted condition compared to the case of the batch method (cf., Ref. 30). In addition, the FIA technique provides precise data with a good reproducibility under a given set of operating conditions. Contribution of these factors, accordingly, leads to the very low detection limit.

The hydrogen peroxide content in rainwater collected in the Tokyo area was determined by this method over the range of 7×10^{-7}–4.3×10^{-5} M (Table 6). The recovery tests were made using 1.00×10^{-6} M hydrogen peroxide with the satisfactory results of 99–102%, indicating the high reliability of the present method.[30]

2. Measurement of Gaseous Hydrogen Peroxide. Atmospheric hydrogen peroxide plays a critical role in the conversion of SO_2 to H_2SO_4.[4,5] To determine atmospheric hydrogen peroxide whose concentrations are of the level of about 2 ppbv, a highly sensitive analysis method with high specificity for hydrogen peroxide is required. Dasgupta and his colleagues had attained the requisite sensitivity only by fluorometric methods with enzymatically mediated reactions in the measurements of gaseous hydrogen peroxide.[38] In these methods, special procedures had still been necessary to prevent interference from concurrently present organic peroxides.

Recently, Li and Dasgupta have paid attention to our Ti–TPyP reagent, and consider it as a promising reagent to advance their studies. They succeeded in showing that the Ti–TPyP reagent was effective to measure ambient levels of gaseous hydrogen peroxide with a light emitting diode (LED)-based liquid-core waveguide (LCW) absorbance detector. Because of the absence of interference from organic peroxides, from SO_2 and from O_3, the detection limit of tens pptv could successfully be attained.[39]

The collection/analysis system and the LED-based LCW absorbance flow cell fabricated by them are shown in Figs. 7 and 8. The diffusion scrubber (DS) is used for collecting gaseous hydrogen peroxide into the liquid phase. An air pump (AP) draws sample air and H_2O_2-free air to the DS. The sampling and H_2O_2-free modes are alternated by switching a three-way valve (V) automatically at the regulated time intervals, and consequently flow injection type signals are obtained.

Water and the reagent solution are aspirated with a peristaltic pump (P) and merged in a tee. The stream flows through a reaction coil and then the LCW absorbance cell. The light (450 nm) transmitted through the cell is coupled to the detector photodiode (PD) by the distal fiber optic.

Methyl hydroperoxide (MHP) is the most common atmospheric organic peroxide. The commonly used enzymatically mediated peroxide assays cannot differentiate between hydrogen peroxide and MHP, since both behave as peroxidatic oxidants. Since the Ti–TPyP reagent does not work on a redox principle, it showed very different behavior. An injection of MHP into the analytical system showed no response at all, suggesting that the Ti–TPyP reagent should be essentially specific for H_2O_2 among peroxides.[39] (In the course of our studies, we also found that benzoyl peroxide had no influence on the Ti–TPyP reagent.)

Effects of SO_2 and O_3 will lead to serious errors in the hydrogen peroxide assay. The former will interfere negatively through its fast redox reaction with hydrogen peroxide. The interference from the latter is more complicated: it reacts with bulk water generating hydrogen peroxide, the reaction being promoted by various surfaces and is also catalyzed by OH^-.[40] However, they found that there was practically no interference from concurrently present gaseous SO_2 and O_3 using a Nafion membrane collector in the DS.[38,39]

The observed absorbance at 450 nm was linear with gaseous hydrogen peroxide concentration at least up to 5 ppbv. The typical system output is shown in Fig. 9. The detection limit was 26 pptv at $S/N = 3$. Owing to the work by Li and Dasgupta, the Ti–TPyP reagent was thus shown to be sufficiently sensitive and selective to measure ambient levels of gaseous hydrogen peroxide, and to be adequate for real atmospheric measurements.[39]

3. Determination of Components and Additives in Foods. Enzymatic methods using commercial kits have become common for determining several components in foods. However, because of high costs of enzymes and difficulties in the continuous measurements, these methods do not appear

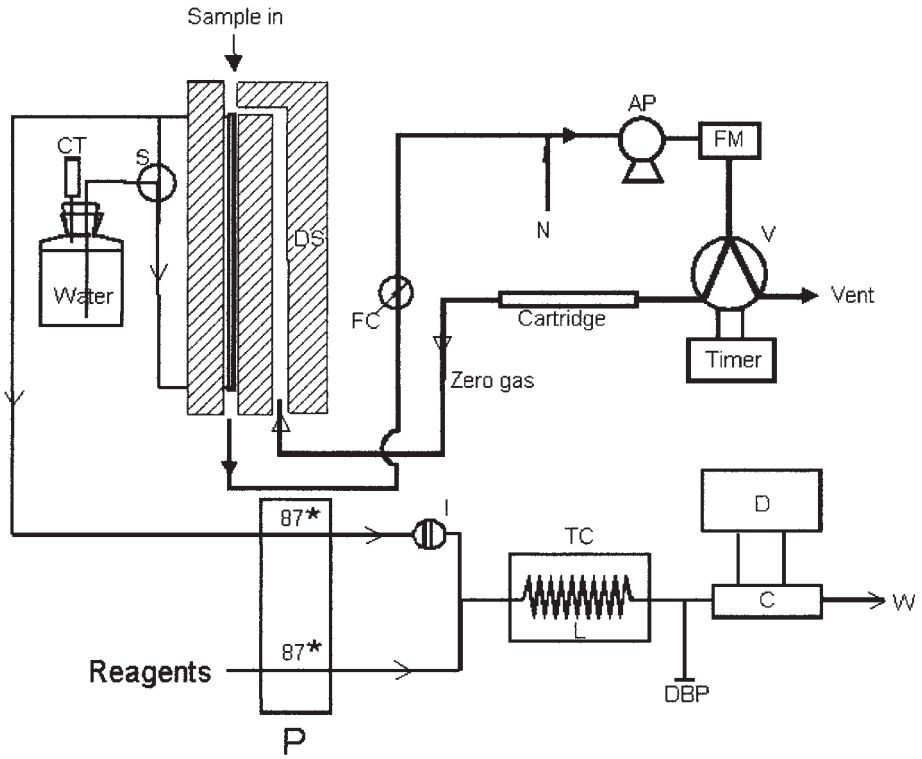

Fig. 7. Schematic diagram of the analytical system. DS: diffusion scrubber; P: peristaltic pump; AP: air pump; V: three-way valve; Cartridge: packed with activated carbon; I: six-port injection valve; L: PTFE reaction coil 0.46 × 3000 mm; TC: temperature controlled reactor enclosure; C: LCW absorbance cell; D: absorbance detector electronics; DBP: debubble port (normally closed); W: to waste; FC: air flow control knob; CT: charcoal trap for protecting reagent; S: 3-way valve for bypassing DS; N: 23 gauge hypodermic needle. ∗ Flow rate of water and the reagent solution: μL min^{-1}.[39]

to be suitable for routine tests. The present FIA method using the Ti–TPyP reagent incorporating with an immobilized enzyme reactor to yield hydrogen peroxide seems promising for developing a new technique in food analysis.

Oxalate:[35] Oxalate exerts undesirable effects on food-manufacturing processes. It adversely affects the food taste, and forms insoluble salts with calcium ions present in water to cause turbidity in beer and fruit juices.[41] Sometimes it causes a renal calculus in the human body.[42] Common methods for determining oxalate in foods include titration by potassium permanganate,[43] ion chromatography,[44] gas chromatography[45] and enzymatic analysis.[46] Among them, enzymatic analysis methods are becoming popular. The methods are based on the detection of hydrogen peroxide or NADH produced through the reaction with the respective enzymes. A kit for oxalate assay by measuring the absorbance of NADH produced with dehydrogenase[47] has been marketed. However, the NADH method is liable to be affected by reducible substances in food, and the procedure is somewhat complicated.

Since the determination of oxalate is thus important in view of food chemistry, application of the present FIA method in combination with the immobilized oxalate oxidase reactor was examined. A schematic diagram of the FIA system is shown in Fig. 10. The carrier solution (CS) was a 0.05 M succinate buffer (pH 3.0). The test solution was prepared according to the procedure of Hansen et al.,[47] and injected using a 20 μL sample loop into the carrier stream. By passing through an immobilized oxalate oxidase column (EC), oxalate in the test solution was converted to hydrogen peroxide through the following enzymatic reaction:

$$\text{Oxalate} + O_2 \xrightarrow{\text{oxalate oxidase}} 2CO_2 + H_2O_2 \qquad (1)$$

The resulting hydrogen peroxide reacted with the Ti–TPyP reagent in the mixing coil to form the peroxo complex, which was detected at 450 nm.

To prepare the enzyme column, oxalate oxidase (7U) was immobilized on Sepharose in a usual way, and packed in a Teflon tube (3 cm long, 2 mm i.d.). The column could be used continuously for more than 200 runs over a period of 8 h. When the column was stored in a refrigerator at 4 °C, no significant decrease in enzyme activity was observed, even after 6 months.

Typical flow signals obtained for the standard oxalate solutions are shown in Fig. 11. Response was linear against the oxalate concentration ($r = 0.999$), in the range from 5.0×10^{-7} to 2.5×10^{-4} M (10–5000 pmol per 20 μL injection). The RSD was 0.48% (2.5×10^{-5} M, $n = 10$).

The method was applied to the determination of oxalate in different kinds of foods such as vegetables, fruits and beverages. The results are listed in Tables 7 and 8, together with the results obtained by the conventional method using an F-kit currently available on the market, for comparison. The two data sets were in good agreement with each other. Recovery tests using the standard oxalate spiked in each test solution were made and the results were in the range of

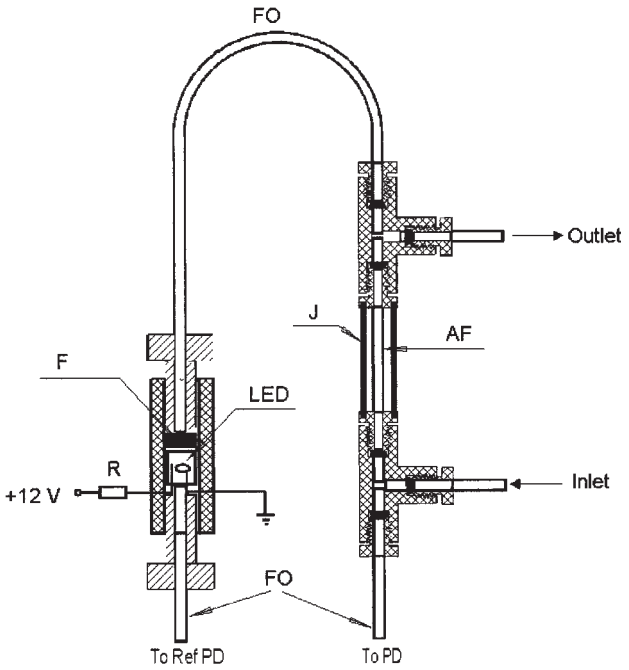

Fig. 8. LED-based LCW absorbance flow cell. FO: Optical fibers, 1.3 mm in diameter, are used to connect the LED with the LCW cell, and the transmitted light from the LCW to the detector photodiode (PD). The reference light from the bottom of the LED is similarly connected to the reference photodiode (Ref PD); F: interference filter; AF: Teflon AF-2400 tube; J: Stainless steel tube jacket; R: current limiting resistor.[39]

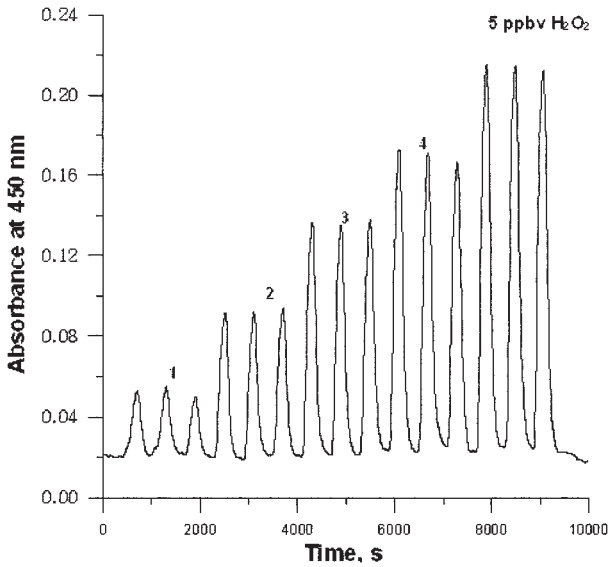

Fig. 9. Typical system output for response to standard H_2O_2 gas from 1 to 5 ppbv, sampling flow rate 1.6 L min^{-1}. Final composition 3 μM Ti–TPyP, 1.6 M $HClO_4$, room temperature; 3/7 min. sample/zero cycles.[39]

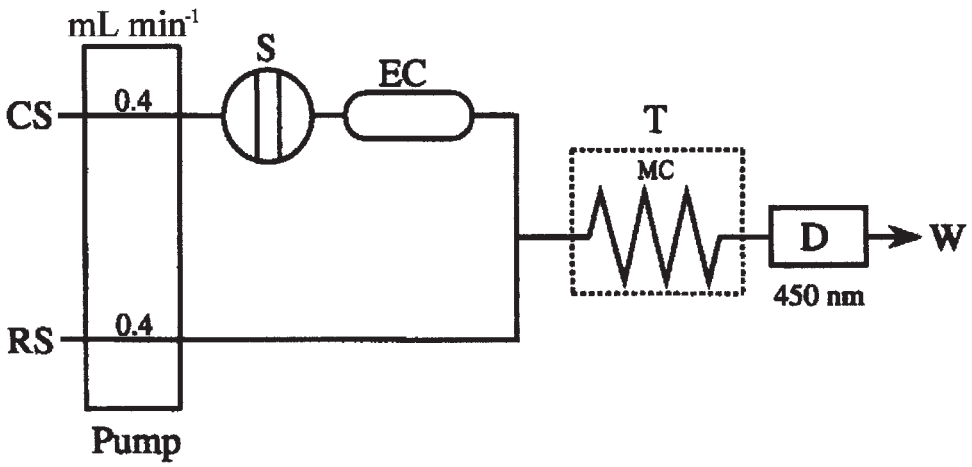

Fig. 10. Flow diagram of the FIA system for oxalate determination using an immobilized oxalate oxidase column reactor. CS: 0.05 M succinate buffer (pH 3.0); RS: 30 μM Ti–TPyP reagent; S: 6-way sampling valve (20 μL); EC: oxalate oxidase column reactor; T: thermostat bath (75 °C); MC: mixing coil (0.5 mm i.d., 15 m long); D: spectrophotometer (450 nm).[31]

97.7–103.0%, indicating the reliability of the analytical data obtained by the present method. The present method was thus shown to be practical and useful for determining oxalate in foods.

Sulfite:[32] Sulfite is commonly used as an additive in food-manufacturing and preserving processes due to its antioxidant, antiseptic and antibacterial abilities. However, it has become apparent that sulfite is liable to cause undesirable effects on the human body as an allergic substance,[48] and consequently, the allowable amounts of sulfite added in each food product are officially regulated.

Among the methods employed so far for determining sulfite in foods,[49–51] the Rankin method[50] is most commonly used; in this method, sulfuric acid formed through the oxidation of sulfite is determined by alkali titration. Although this method allows the detection of sulfite in ppm levels, the procedure involves the use of a particular piece of distillation equipment and is somewhat too complicated to be suited to practical uses.

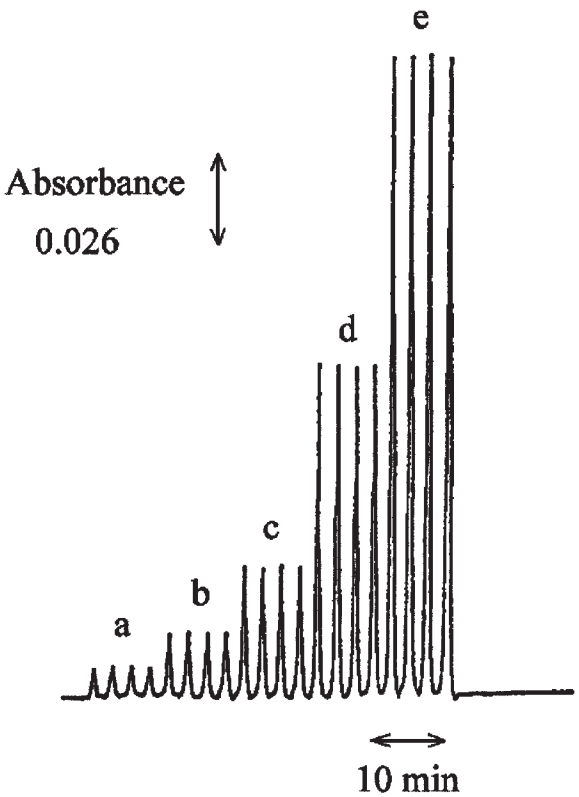

Fig. 11. Typical response signal for oxalate. Each injection volume: 20 μL. Oxalate concentration: a, 5; b, 10; c, 20; d, 50; e, 100 μM.[31]

A simpler, more rapid and sensitive determination of sulfite was realized by the present FIA method in combination with an immobilized sulfite oxidase reactor to yield hydrogen peroxide through the following reaction:

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

The FIA system was essentially the same as that shown in Fig. 10. In this case, a Teflon-tube (6 cm long, 2 mm i.d.) packed with sulfite oxidase-bearing Sepharose was used as an enzyme column. The carrier solution was a Tris–HCl buffer of pH 8.2, and 5 μL of the test solution was injected into the carrier stream. Other FIA operation conditions were the same as those described in the oxalate assay. The peak height of the FIA flow signal showed a good linear relation against the sulfite concentration ($r = 0.999$) in the range of 1.0×10^{-6} to 5.0×10^{-4} M (5 to 2500 pmol per 5 μL injection), and the RSD value was 0.53% ($n = 10$, 1.0×10^{-4} M). By this method, amounts of sulfite in different kinds of foods, such as wines, fruit juices, and dry and frozen foods, were determined. A part of the results are shown in Table 9 . Because of high sensitivity, very small amounts of naturally occurring sulfite contained in fruit juices could be determined by this method.

Sugars:[33] Determination of sugars is essential for the quality and process assessment in food manufactures. For determining sugars, chromatographic techniques such as GC[52] and HPLC,[53] enzymatic method[54] and FIA method[55–57] are commonly employed. Among them, FIA method incorporated with an enzyme reactor seems convenient as a simple and rapid means, however, with this method it is rather difficult to determine each constituent sugar continuously.

The present FIA method with several enzyme reactors arranged in parallel, in which each flow line was chosen by a

Table 7. Oxalate Content in Vegetables and Fruits and the Recovery Values of Added Oxalate Obtained by the Present Method and the F-kit Method[31]

Sample	Present method		F-kit method
	Oxalate content mg/100 g	Recovery* /%	Oxalate content mg/100 g
Spinach	515	101.9	540
Japanese radish	1.46	103.0	
Ginger	203	99.3	177
Japanese ginger	225	101.8	206
Kiwi fruit	4.50	100.0	
Persimmon	2.61	99.0	
Millet Jelly	4.53	101.3	

∗ [Oxalate] added: 9.00×10^{-4} g/L.

Table 8. Oxalate Content in Beverages and the Recovery Values of Added Oxalate Obtained by the Present Method and the F-kit Method[31]

Sample	Present method		F-kit method
	Oxalate content /mg L^{-1}	Recovery* /%	Oxalate content /mg L^{-1}
Beer 1	10.4	97.7	
Beer 2	3.29	97.7	
Beer 3	7.56	100.6	
Beer 4	18.0	102.0	
Beer 5	9.72	103.0	
Beer 6	18.9	104.0	20.8
Apple juice	4.77	100.8	
Orange juice	1.80	101.5	
Tomato juice	42.3	97.7	
Carrot juice	21.9	98.6	15.1

∗ [Oxalate] added: 9.00×10^{-4} g/L.

Table 9. Sulfite Content in Beverages and Solid Food and the Recovery Values of Added Sulfite[32]

Sample	Sulfite content /mg kg^{-1}	Sulfite added /mg kg^{-1}	Recovery /%
Red wine 1	129	125	96.6
Red wine 2	156	625	101.5
Red wine 3	135	625	101.4
White wine 1	296	250	99.6
White wine 2	194	250	101.0
Rose wine	184	250	98.6
Orange juice	21.3	62.5	99.7
Grapefruit juice	21.0	62.5	98.8
Apple juice	16.3	62.5	101.4
Raisin	35.8	12.5	100.9
Dry plum	59.6	12.5	96.2
Dry apricot	101	12.5	94.7
Frozen shrimp 1	5.35	40.0	98.4
Frozen shrimp 2	21.4	40.0	97.4

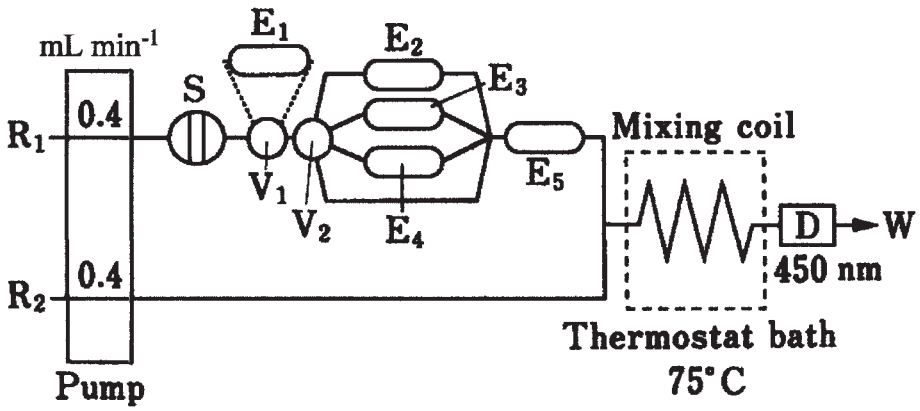

Fig. 12. Flow diagram of the FIA system for sugar determination using immobilized enzyme reactors. CS: 0.05 M phosphate buffer (pH 6.6) containing 1 mM $MgCl_2$; RS: 30 μM Ti–TPyP reagent; E_1: glucose eliminating reactor (glucose oxidase, catalase); E_2: sucrose reactor (β-fructosidase, mutarotase); E_3: maltose reactor (maltase); E_4: lactose reactor (β-galactosidase); E_5: glucose reactor (glucose oxidase); V_1, V_2: switching valve; S: sample loop (20 μL); Mixing coil: 0.5 mm i.d., 15 m long.[33]

switching valve, permitted the continuous determination of glucose, sucrose, maltose and lactose.[33] A flow diagram of the FIA system thus fabricated is shown in Fig. 12. A phosphate buffer (0.05 M, pH 6.6) containing 1 mM $MgCl_2$ was served as a carrier solution. Test solution (20 μL) was injected using a sample loop. β-D-Glucose in the test solution was oxidized to form hydrogen peroxide quantitatively by passing through the glucose reactor (E_5) packed with glucose oxidase. Sucrose, maltose and lactose (disaccharides) in the test solution were hydrolyzed to form β-D-glucose through their corresponding hydrolysis reactors (E_2, E_3 and E_4, respectively), and the resulting β-D-glucose was converted continuously to hydrogen peroxide through the glucose reactor (E_5). Prior to the determination of sucrose, maltose and lactose, glucose in the test solution was removed by passing through the glucose-eliminating reactor (E_1) using a switching valve V_1. In the reactor E_1, glucose oxidase and catalase were packed. In all cases, the effluent hydrogen peroxide from the glucose reactor (E_5) was mixed with the Ti–TPyP reagent in the mixing coil, and detected by the measurement of the absorbance at 450 nm.

The enzyme mediated reactions for the four sugars taking place in reactors E_2–E_5 are as follows:

Sucrose reactor (E_2):

$$\text{Sucrose} + H_2O \xrightarrow{\beta\text{-fructosidase}} \alpha\text{-D-Glucose} + \text{D-Galactose} \quad (3)$$

$$\alpha\text{-D-Glucose} \xrightleftharpoons{\text{mutarotase}} \beta\text{-D-Glucose} \quad (4)$$

Maltose reactor (E_3):

$$\text{Maltose} + H_2O \xrightarrow{\text{maltase}} \alpha\text{-D-Glucose} + \beta\text{-D-Glucose} \quad (5)$$

Lactose reactor (E_4):

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \beta\text{-D-Glucose} + \text{D-Fructose} \quad (6)$$

Glucose reactor (E_5):

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \beta\text{-D-Gluconic acid} + H_2O_2 \quad (7)$$

Table 10. Sugar Content in Milk and Beverages[33]

Sample	Content, g/100 g			
	Glucose	Sucrose	Maltose	Lactose
Milk	0.00785	0.00243	N.D.	5.40
Cream powder	1.44	0.589	0.137	46.1
Lactobacilli beverage 1	0.645	7.56	0.93	3.75
Lactobacilli beverage 2	4.20	6.33	1.62	2.40
Milky beverage	3.62	2.88	0.749	3.71
Soft drink	1.30	0.100	0.0548	N.D.
Fruit juice	2.31	1.73	0.0951	N.D.
Malt tea	0.00371	N.D.	0.00667	N.D.
Wine	0.818	N.D.	N.D.	N.D.
Sake	1.42	N.D.	0.0883	N.D.
Beer 1	0.0133	N.D.	0.195	N.D.
Beer 2	0.00775	N.D.	0.171	N.D.

Linear relations were obtained between the peak heights of the flow signals and the sugar concentrations in the ranges of 5.0×10^{-7}–5.0×10^{-4} M (10–10000 pmol per test) for glucose, and 1.0×10^{-6}–1.0×10^{-3} M (20–20000 pmol per test) for sucrose, maltose and lactose. The RSD values for the ten replicate determinations of glucose, sucrose, maltose and lactose using each standard solution of 1.0×10^{-4} M were 0.74, 0.75, 0.84 and 0.49%, respectively. The operation conditions of this method permitted analysis of 20 kinds of sugars per hour. The glucose reactor was stable for more than 6 months, while other reactors were only available for about 1 month when processing test solutions.

The method was applied to the determination of these four sugars in beverages such as milk, soft drinks, wine and beer. A part of the results are listed in Table 10. By this method, the sugar content in beverages was determined rapidly up to the level of 0.1 ppm. Results of the recovery tests were 95–105%, indicating the high reliability of the present method.

Free Fatty Acid Content in Oils:[58] Oils tend to decompose slowly upon storage in contact with the atmosphere and

to release their fatty acid constituents. Accordingly, a quality and freshness assessment of oils can be made based on the determination of the free fatty acid content. The present FIA system was also found to be effective for determining the free fatty acid content in oils using immobilized acyl CoA synthetase (ACS) and acyl CoA oxidase (ACO) reactors connected in series, in which free fatty acids (FFA) were converted to hydrogen peroxide through the following reactions:

$$\text{FFA} + \text{CoA} + \text{ATP} \xrightarrow{\text{ACS}} \text{acyl CoA} + \text{AMP} + PPi \quad (8)$$

$$\text{acyl CoA} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{ACO}} \text{2,3-}transenoyl\ \text{CoA} + \text{H}_2\text{O}_2 \quad (9)$$

The FIA system was essentially the same as that shown in Fig. 10. Tris–HCl buffer of pH 8.5 containing $MgCl_2$, ATP and poly(oxyethylene)(10) octyl phenyl ether served as a carrier solution. Poly(oxyethylene)(10) octyl phenyl ether was used to accelerate the above enzymatic reactions.[59] During the preparation of the test solution, sample oil was dissolved in a small amount of poly(oxyethylene)(10) octyl phenyl ether, followed by adding CoA and then diluting with distilled water.

The peak height of the flow signal showed a good linear relation in the range of 1.0×10^{-6} to 1.0×10^{-4} M (20–2000 pmol per 20 μL injection) for palmitic, oleic and linoleic acids. No appreciable difference in the FIA peak height was detected for each injection of 1.0×10^{-4} M palmitic, oleic and linoleic acids, suggesting that both ACS and ACO enzymes showed nearly the same reaction efficiency for these acids.

The method was actually applied to determine the free fatty acid content in 7 kinds of plant oils (camellia, olive, soy bean, sesame, peanut, rape seed and corn oils). A commonly used method for determining the free fatty acid content in oils is alkali titration.[60–63] Comparison of the content data obtained by the present method with those obtained by the conventional titration method showed excellent correlation ($r = 0.999$), suggesting that the present method will be useful for the determination of the free fatty acid content in oils (Fig. 13).

The present method was found to be empirically valid for the total acid content in oils, provided the enzyme reaction efficiencies are nearly the same for each acid. The application of this method to the determination of the total amounts of free fatty acids in edible plant oils seems a favorable choice, because their constituent acids are restricted to higher monocarboxylic acids of C_{16}–C_{20}. For extensive applications of the method to various kinds of fats and oils, further examination about the reaction efficiencies of the enzyme reactors will be necessary.

4. Determination of Biological Components in Human Serum and Urine. In biochemical analysis, various enzymatic techniques have been commonly employed to determine the desired components in biological samples such as blood and urine. In general, the components are oxidized through enzymatically mediated processes to produce hydrogen peroxide. The determination of hydrogen peroxide in high sensitivity is thus important in this regard. The use of the Ti–TPyP reagent was extended to the determination of glucose and uric acid in human serum and urine to show the effectiveness of the reagent in clinical and biochemical analysis.

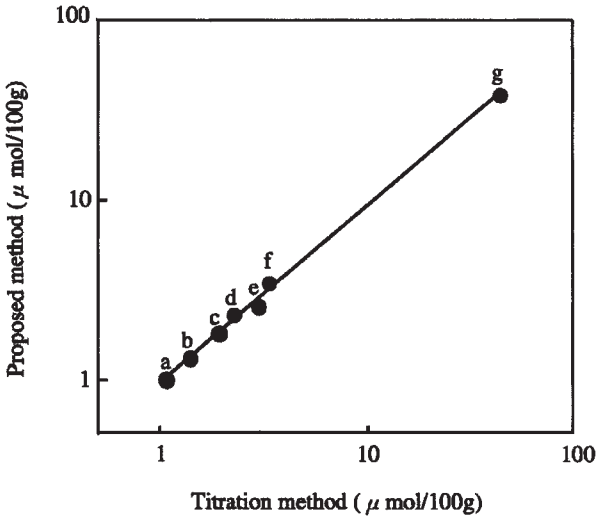

Fig. 13. Correlation between the free fatty acid content in plant oils obtained by the conventional titration and the present methods. Oil samples: a, soy bean oil; b, rape seed oil; c, peanut oil; d, sesame oil; e, olive oil; f, corn oil; g, camellia oil.

Glucose:[34,35] Since blood glucose assay is most frequently made in clinical tests, development of a new analysis method that required only a small sample size is highly desirable. A highly sensitive method is also required for urine glucose assay, because the glucose content in urine is lower than that in blood.

Use of the Ti–TPyP reagent was examined for the determination of glucose in human serum and urine combined with the following enzymatic process of glucose oxidase (GOD),[35]

$$\beta\text{-D-Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{D-Gluconic acid} + \text{H}_2\text{O}_2 \quad (10)$$

where the resulting hydrogen peroxide was determined based on the absorbance change at 432 nm (ΔA_{432}). The ΔA_{432} value showed a linear relation against the glucose concentration ranging from 2.0×10^{-8} to 3.2×10^{-6} M (50 pmol–8 nmol per test). The apparent molar absorptivity of glucose was $1.8 \times 10^5\ \text{M}^{-1}\,\text{cm}^{-1}$.

Determinations of glucose in human serum and urine were actually made by the present method. To prepare the test solutions, we diluted only 1 μL of serum or urine with water in 200- or 50-fold, respectively. No complicated pretreatment procedure of samples, such as deproteinization and preconcentration, was needed. To confirm the validity of the method, we performed the determination of glucose for commercially available "control serums." Both "normal" and "abnormal" control serums obtained from Nihon Shoji Co. were used. (Glucose content was indicated for each control serum). Compared to the indicated values, obtained results were reasonable for both normal serum (content data obtained: 110–119 mg dL^{-1}) and abnormal serum (166–249 mg dL^{-1}). Content data of glucose in human urine (6.54–90.8 mg dL^{-1}) was also obtained by this method. In all cases, no preconcentration pro-

cedure was required because of the high sensitivity of the Ti–TPyP reagent for hydrogen peroxide. The method was thus shown to be useful to determine glucose levels not only in blood but also in urine, even though the latter levels were lower than those in the former.

The presence of foreign substances in samples, such as inorganic cations and anions, ascorbic acid and amino acids, had no influence on the analytical results to the extent of normal amounts present in serum and urine. Content of ascorbic acid in serum is about one-hundredth of that of glucose. The effect of ascorbic acid was found to be practically negligible unless its amount exceeded one-tenth of glucose content.

More sensitive and rapid analysis of glucose in serum was achieved by the FIA technique incorporated with a GOD reactor and the Ti–TPyP reagent.[34] The FIA system was basically the same as that shown in Fig. 10, except for using an enzyme reactor containing immobilized GOD.[64,65] As a carrier solution, 0.05 M phosphate buffer (pH 6.0) was used. Determination of glucose in a test solution containing the standard glucose was made by measuring the peak height of the flow signal at 450 nm, based on the apparent molar absorptivity for hydrogen peroxide (ε_{450}) as described above. Response was linear against the glucose concentration in the range of 1×10^{-6}–1×10^{-3} M (5 pmol–5nmol per test), and the detection limit was 2.5 pmol per test. The RSD value was 0.64% (1×10^{-4} M, $n = 10$). Throughput rate was 40 samples per hour.

The method was applied to the determination of human blood glucose using control serums (normal and abnormal serums obtained from Nihon Shoji Co.). In this case, 0.05 μL control serum was diluted with water in 100-fold, and served as a test solution. A 5 μL aliquot of the test solution was injected into the carrier stream. The results were consistent with the content values indicated for the control serums. The results showed a good correlation with those obtained by the conventional method using a commercial kit ("Glucose B Test Wako", Wako Pure Chemical Industries, based on the GOD, POD and 4-aminoantipyrine–phenol method), as shown in Fig. 14.

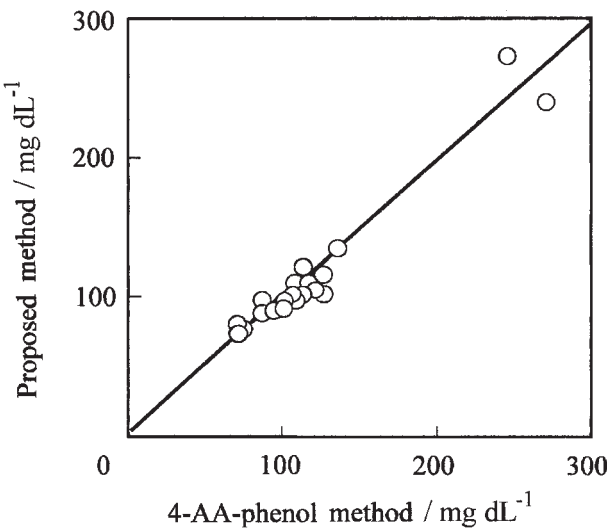

Fig. 14. Correlation between the serum glucose levels obtained by the glucose oxidase–4-aminoantipyrine–phenol and the present methods.

Uric acid:[34,36] Determination of uric acid in human serum is important as an indication of several diseases such as gout. Because of its lower content in serum compared to that of glucose or cholesterol, highly sensitive and selective analysis method is particularly required in the clinical field. In general, uric acid is analyzed by treating the samples with uricase enzyme to form hydrogen peroxide. To detect the resulting hydrogen peroxide, colorimetric methods incorporated with uricase–catalase[66] or uricase–peroxidase[67] mediated oxidation of a colorless chromogen to form a colored product are commonly used. However, these methods are not necessarily sufficient to fill the demand in sensitivity and selectivity. Although chemiluminescence measurement provides a very sensitive method,[68] this method is often susceptible to the presence of other substances.

Then application of the Ti–TPyP reagent was extended to this object, in which 5 μL of serum sample was incubated with an uricase solution (0.05 U mL^{-1}) to produce hydrogen peroxide through the following enzymatic oxidation:[36]

$$\text{Urate} + 2H_2O + O_2 \xrightarrow{\text{uricase}} \text{Allantoin} + H_2O_2 + CO_2 \quad (11)$$

After adding the Ti–TPyP reagent to the reaction mixture, we determined the produced hydrogen peroxide by the absorbance measurement at 432 nm. A linear relation was obtained between the ΔA_{432} and the uric acid concentration in serum in the range of 5.0×10^{-6}–1.0×10^{-3} M. This range corresponds to that of 0.08–16.8 mg dL^{-1}, indicating that the present method will be suited for routine tests in the clinical fields, since uric acid levels in serum are ca. 2.4–7.5 mg dL^{-1} for healthy adults.

The FIA method incorporated with an uricase reactor was also applied to determine uric acid in serum.[34] As a carrier solution, 0.2 M borate buffer (pH 7.2) was used. In this case, 0.5 μL serum was diluted with water in 10-fold, and injected into the carrier stream.

As shown in Fig. 15, analytical data obtained for 21 human

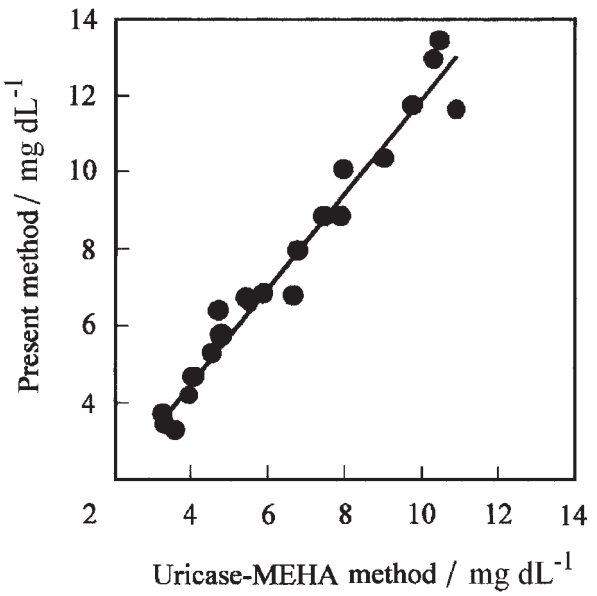

Fig. 15. Correlation between the serum uric acid levels obtained by the uricase–MEHA and the present methods.[36]

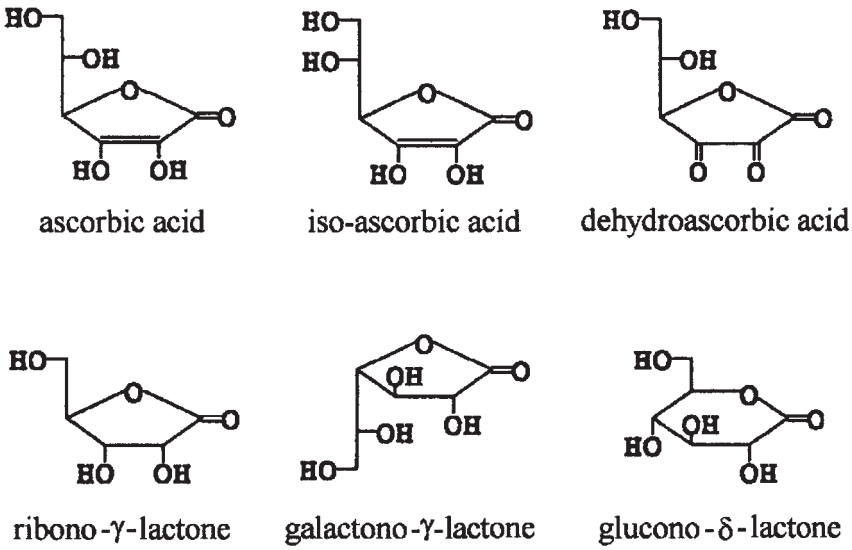

Fig. 16. Ascorbic acid and its analogous compounds.

serum samples showed a good correlation ($r = 0.982$) against those obtained by the conventional method using a commercially available kit (Uric acid B Test Wako, Wako Pure Chemical Industries, based on the uricase and 3-methyl-*N*-ethyl-*N*-(2-hydroxyethyl)aniline (MEHA) method), however, the kit method needed 50 μL of deproteinized serum, while the present method required a far smaller sample size, as stated above. Because of high sensitivity and selectivity of the Ti–TPyP reagent, neither preconcetration nor deproteinization of the serum samples was required.[34,36]

Others: Besides glucose and uric acid, the Ti–TPyP reagent can be used for the determination of a variety of analytes in clinical and biochemical assays, by treating the samples with the corresponding oxidase enzymes. Using this reagent, the determination of galactose and 3-hydroxybutyric acid in human serum was made with satisfactory results.[34] Because of very low content in serum, these components are generally known to be difficult to determine by the conventional methods, however, the Ti–TPyP reagent facilitated the determination owing its high sensitivity and selectivity for hydrogen peroxide.

Catalytic Behavior of the $TiO(tpypH_4)^{4+}$ Complex to Ascorbate

As described above, interference from ascorbic acid present in the test solutions was apparent during the course of the applications of the Ti–TPyP reagent to the real samples. The presence of ascorbate over one-tenth part of hydrogen peroxide led to a considerable positive error. This was really an unexpected fact at the beginning of the present studies. Occurrence of the error is considered to be due to the undesirable production of hydrogen peroxide through the catalytic (enzyme-like) action of the $TiO(tpypH_4)^{4+}$ complex to ascorbate, similarly to the catalytic effects of certain metal complexes.[69]

In our absorption measurements of ascorbic acid in the presence of the Ti–TPyP reagent, ascorbate was found to react with $TiO(tpypH_4)^{4+}$ complex upon heating in strong acid solution to form its peroxo complex, $TiO_2(tpypH_4)^{4+}$, implying that the catalytic production of hydrogen peroxide was involved in the reaction pathway.[70] Similar findings were also observed for iso-ascorbic acid. Contrary to this, dehydroascorbic acid, ribono-γ-lactone, galactono-γ-lactone and glucono-γ-lactone did not react with the Ti–TPyP reagent at all, indicating that the ene-diol moiety in the lacton ring is essential for such catalytic oxidation (Their molecular structures: in Fig. 16). In addition, the reaction was promoted by dissolved oxygen in the solution and irradiation of visible light.

These findings suggest that a radical formation process is responsible for the oxidation of ascorbic acid, similarly to the catalytic oxidation of ascorbic acid with $Ru(bpy)_3^{2+}$ complex to form hydrogen peroxide.[69] Further details of this reaction mechanism are not yet completely elucidated.

Concluding Remarks

In this account, the Ti–TPyP reagent is shown to be quite useful as a highly sensitive and selective spectrophtometric reagent for determining traces of hydrogen peroxide.

The degree of the absorbance decrease at 432 nm per 1 M hydrogen peroxide (A_{432}) is 1.9×10^5 $M^{-1}\,cm^{-1}$, while the apparent molar absorptivity for hydrogen peroxide at 450 nm (ε_{450}) is 1.1×10^5 $M^{-1}\,cm^{-1}$. We have thus attained the goal of the molar absorptivity of the order of 10^5 $M^{-1}\,cm^{-1}$. In the batch method, absorption measurement at 432 nm is recommended to determine hydrogen peroxide. The detection limit is 25 pmol per test by this method. The sensitivity of this method exceeds that of the conventional peroxidase (POD)–4-aminoantipyrine–phenol method by 16 times.[71]

Furthermore, we have attained higher sensitivity using the Ti–TPyP reagent coupled with FIA method with the detection limit of 0.5 pmol per test. In this case, absorbance measurement at 450 nm is preferable to detect the peroxo complex. Comparing with the detection limit of 78 pmol per test obtained by FIA combined with the POD method,[6] the present method is shown to far surpass the conventional one in a point of its sensitivity. Accordingly, the present method requires smaller sample size. In addition to the sensitivity, the FIA method using the Ti–TPyP reagent is superior to the conventional method in terms of accuracy, rapidity and simplicity.

No sophisticated equipment is needed.

Because of great affinity of the Ti–TPyP reagent for hydrogen peroxide, most foreign substances (inorganic, organic substances involving organic peroxides) concurrently present in real samples cause practically no interference on the present method. Consequently, no complicated pretreatment of samples, such as preconcentration and deproteinization, is required prior to the measurements. Even though the reagent has a problem arising from the unexpected effect of ascorbate, its interference can be simply avoided if one uses ascorbate oxidase. Sometimes it can be more simply neglected only by diluting the test solution, owing to the high sensitivity of the reagent.

Such outstanding characteristics of the Ti–TPyP reagent have facilitated its wide applications to the determination not only of traces of hydrogen peroxide itself but also of a variety of components in real samples using appropriate oxidase enzymes. We believe that the Ti–TPyP reagent has a potential for further extension of applications besides those described in this account. Recently, we have succeeded in the enzyme activity determination by the FIA method using this reagent. Use of the reagent will be extended to the measurement of ammonia content for freshness or putrefaction assessment of foods.

The present FIA method still seems to contain some points to be further improved. Treatment of strong acid in the waste fluids from the flow injection system was easily made by neutralization, accompanying concurrently the removal of titanium as precipitates. Regarding to such subjects, Dasgupta has pointed out in his recent report[39] about the possibilities of the recycle of the acid by using a cation exchange resin column from the finding of a great affinity of the Ti–TPyP reagent for cation exchange resins, and the regeneration of the reagent from the Ti–TPyP–H_2O_2 complex by destroying the H_2O_2 through an appropriate catalyst like platinum black. Such proposal is worthy of a further investigation with regard to the possibility of measuring the Ti–TPyP–H_2O_2 complex on the resin bed.

Since our analytical studies using the Ti–TPyP reagent was initiated, several water-soluble porphyrins having larger molar absorptivitiy have been developed. Further investigation is to be expected to develop more intensely absorbing titanium complexes for detecting hydrogen peroxide in the future.

Perhaps "hydrogen peroxide" may sound like a very old and unfashionable substance, but it has been a significant analyte for a long time, as the old proverb says "By exploring the old, one becomes able to understand the new." We will be very glad if the readers are interested in some points described in this account.

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