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ORIGINAL PAPER

Fluorophotometric Determination of Hydrogen Peroxide with Fluorescin in the Presence of Cobalt (II) and Reaction Against Other Reactive Oxygen Species

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Abstract A fluorophotometric method for the determination of hydrogen peroxide (H_2O_2) using fluorescin was developed. This method was based on the oxidative reaction of fluorescin, a colorless, non-fluorescent lactoid fluorescein, by H_2O_2 to give highly fluorescein fluorescence emission. In the determination of H_2O_2, the calibration curve exhibited linearity over the H_2O_2 concentration range of 1.5–310 ng mL^{-1} at an emission wavelength of 525 nm with an excitation of 500 nm and with relative standard deviations (n=6) of 2.51%, 2.48%, and 1.31% for 3.1 ng mL^{-1}, 30.8 ng mL^{-1}, and for 308 ng mL^{-1} of H_2O_2, respectively. The detection limit for H_2O_2 was 1.9 ng mL^{-1} six blank determinations was performed (ρ=6). This proposed method was applied to detection of other reactive oxygen species and nitrogen species (ROS/RNS) such as singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), peroxynitrite ($ONOO^-$) etc., and it was possible to detect them with a high sensitivity. In addition, this proposed method was applied to the recovery tests of H_2O_2 in calf serum, human saliva, rain water, and wheat noodles; the results were satisfactory.

Keywords Fluorophotometric determination · Hydrogen peroxide · Reactive oxidant species · Reactive nitrogen species · Fluorescin

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Introduction

Oxidative stress is imposed on cells as a result of one of three factors: 1) the increase in oxidant generation, 2) the decrease in antioxidant protection, or 3) the failure to repair oxidative damage. Cell damage is induced by ROS/RNS such as H_2O_2, $^1O_2$, superoxide (O_2^-), $^{\bullet}OH$, nitric oxide (NO), $ONOO^-$, and alkylperoxyl radical ($ROO^{\bullet}$). ROS/RNS have been identified as important chemical mediators that found to affect living bodies and cause various types of diseases such as aging, cancer [1], diabetes mellitus, atherosclerosis, cardiovascular disorders [2], and neurological degeneration such as Alzheimer's disease and schizophrenia. Whereas, ROS fulfil important prerequisites for intracellular messenger molecules [3–5]. In addition, H_2O_2 is a major by-product of ROS in living organisms and a common marker for oxidative stress in the field of clinical inspection [6–8]. The development of methods for the determination of the level of these substances is significant in the fields of clinical and biological studies. ROS/RNS have been measured by chemiluminescence [9, 10], spectrophotometry [11–13], flow cytometry [14, 15], and fluorophotometry [16–22], have already been reported. These methods focus on the determination of individual ROS/RNS levels. However, it appears that most of ROS/RNS have lifetimes of only a few minute under biological environments. Therefore, we developed a handy and highly sensitive method for the determination of total ROS/RNS levels including those of H_2O_2 to determine ROS/RNS effectively as an oxidation stress marker in the clinical field. We have already developed methods for the fluorometric determination of Co (II) and H_2O_2 levels with fluorescein hydrazide (FH) [23, 24]. In this study, the standard procedure was more simplified and the effect of foreign substances was

relatively improved. This proposed method was able to completely detect the H_2O_2, $^1O_2$, $^{\bullet}OH$, and $ONOO^-$ levels. The proposed method was then applied to the recovery tests of H_2O_2 in calf serum, human saliva, rain water, and wheat noodles.

Experimental

Reagent and solutions

A fluorescin solution (4.0×10^{-6}mol L^{-1}) was prepared by dissolving fluorescin (Tokyo Chemical Industry Co. Ltd.) in ethanol. A cobalt (II) solution (1.0×10^{-4}mol L^{-1}) was prepared by dissolving cobalt (II) chloride, 6-hydrate (Kishida Chemical Co. Ltd.). A 0.2 mol L^{-1} Tris-HCl (tris (hydroxymethyl) aminomethane hydrochloride) buffer (pH 8.5) was used for adjusting the pH values. A stock solution of 1.0×10^{-2}mol L^{-1} H_2O_2 was prepared by diluting a 30% H_2O_2 solution (Kishida Chemical Co. Ltd.) and correcting by permanganimetry, and a working solution was prepared by the suitable dilution of this stock solution as required. $^1O_2$ was generated by a sodium molybdate dehydrate (Na_2MoO_4)/H_2O_2 system. Na_2MoO_4 was purchased from Sigma Aldrich. O_2^- was generated by the reaction of hypoxanthine (Wako Pure Chemical Industries Co. Ltd.) with xanthine oxidase (Wako Pure Chemical Industries Co. Ltd.). $^{\bullet}OH$ was generated by the reaction of ferrous perchlorate with H_2O_2. Ferrous perchlorate was purchased from Tokyo Chemical Industry Co. Ltd.. Anion hypochlorite (OCl^-) was prepared from sodium hypochlorite (Tokyo Chemical Industry Co. Ltd.). NO was generated by 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminoethyl)-3-methyl-1-triazene (NOC7) (Dojindo Chemical Co. Ltd.). A standard solution of NOC7 was freshly prepared in a 0.1 mol L^{-1} NaOH solution. $ONOO^-$ was prepared by peroxynitrite solution (Dojindo Chemical Co.). $ONOO^-$ levels were determined by using UV at 300 nm. $ROO^{\bullet}$ was generated by 2, 2′-azobis(2-amidinopropane)dihydrochloride (Wako Pure Chemical Industries Co. Ltd.). De-ionized water was used throughout the experiment. All the materials and reagents were of analytical grade and were used without further purification.

Apparatus

Fluorescence measurements were performed on a Hitachi model F-2500 spectrofluorophotometer equipped with an Ushio 150 W xenon lamp, 10×10 mm quartz cells, and a Varioskan fluorescence spectrometer (Thermo Electron). A Horiba model F-22 pH meter equipped with a glass combined electrode was used for all pH measurements.

Standard procedure for the determination of H_2O_2 levels

A H_2O_2 sample (1.5–310 ng) was placed in a 10 mL calibrate flask, and then 2.0 mL of 0.1 mol L^{-1} Tris-HCl buffer solution (pH8.5), 0.5 mL of 1.0×10^{-4}mol L^{-1} cobalt (II) solution, and 0.4 mL of a 1.0×10^{-5}mol L^{-1} fluorescin solution were added. The solution was diluted to 10 mL with water, transferred to a test tube, mixed well, and maintained at 40 °C for 30 min. After cooling at room temperature in water for 5 min, the difference between the relative fluorescence intensities [RFI=(A–B)/B] of solution A and reagent blank (solution B), which were prepared under the same conditions, was measured by examining the excitation/emission wavelength at 500/525 nm.

Results and discussion

Spectral properties

It has been reported that the neutral form of fluorescein can occur in three different tautomers, i.e., zwitterion, quinoid, and lactone; the colorless lactonic form is usually the dominant tautomer that is present in organic solvents [25, 26]. Figure 1 shows the structure of fluorescin and tautomers, fluorescin used in this method is a colorless and nonfluorescent lactoid dye [27]. This nonfluorescent compound when properly activated and in the presence of H_2O_2 and other ROS/RNS is rapidly oxidized to a fluorescent compound. Figure 2 shows the absorption spectra of fluorescein (Fig. 2, curve f), fluorescin and its reaction solution with H_2O_2 (30 ng mL^{-1}, 150 ng mL^{-1}, 300 ng mL^{-1}, and 1,500 ng mL^{-1}) in a 10 mL calibrate flask, 2.0 mL of the Tris-HCl buffer solution (pH8.5), and 0.5 mL of 1.0×10^{-4}mol L^{-1} cobalt (II) solution (Fig. 2, curves b–e). It can be observed that fluorescin is little absorbed in the visible region (Fig. 2, curve a); the molar absorptivity of fluorescin corresponds to the characteristic absorption peak (490 nm, curve F) of fluorescein. This can be attributed to its closed spirolacton form. However, upon reaction with H_2O_2, the green color (Fig. 2, curves b–e), which indicates fluorescein, is noticeably restored. Figure 3 presents the fluorescence emission spectra of a mixture of fluorescin with cobalt (II) as a blank solution (Fig. 3, curve a) that was obtained after H_2O_2 was added to the blank solution as a sample solution (Fig. 3, curves b–g). When H_2O_2 was introduced into a solution of fluorescin, fluorescence emission identical to that of fluorescein was observed with the maximum fluorescence emission at 525 nm. Further, the fluorescence development reaction was significantly facilitated when it was performed in the presence of cobalt (II). In order to explore the possible reaction products, the fluorescence emission spectra of the

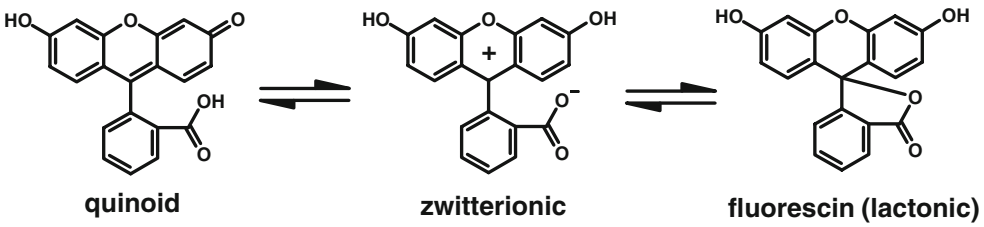

Fig. 1 The structure of fluorescin and its tautomers

reaction solution were compared with that of fluorescein (Fig. 3, curve h). The absorption and emission spectra of the sample solution were observed at pH8.5. The results indicate that these spectra are identical and both have the same values of the maximum emission wavelengths. From the above results, it was assumed that fluorescin was oxidized by H_2O_2 to yield the highly fluorescent product, fluorescein.

Optimization of experimental conditions

Various experimental conditions such as pH, surfactants, metal ions that were used as reaction catalysts, concentration of fluorescin, temperature, and time were examined to optimize the reaction conditions.

The effect of pH on the fluorescence investigated by using various buffer solutions and pH values, such as 0.2 mol L^{-1} Tris-HCl, 0.2 mol L^{-1} $NaHPO_4/NaH_2PO_4$, 0.2 mol L^{-1} glycine/NaOH, and 0.2 mol L^{-1} $NaHCO_3/Na_2CO_3$. The maximum constant value of RFI was observed over a pH range of 8.3–8.8 with 0.2 mol L^{-1} Tris-HCl buffer solution; thus, 2.0 mL of the buffer solution (pH8.5) was used for adjusting the pH values in the final volume of 10 mL.

The addition of various surfactants in the coloring or fluorescence reactions between various reagents and metal ions has already offered many advantages in comparison to the absence of surfactants [28–32]. Accordingly, in order to develop fluorescence and enhance the sensitivity, we examined the effects of different surfactants: cationic [dodecyltrimethylammonium chloride (DTAC), hexadecyltrimethylammonium chloride (HTAC), stearyltrimethylammonium chloride (STAC), hexadecylpyridiniumchloride (HPC), hexadecylpyridinium bromide (HPB), benzyldimethyltetradecyl ammonium chloride (Zephiramine)], anionic [sodium dodecyl sulfate (SDS), di-(2-ethylhexyl) sodium sulfosuccinate (AOT)], nonionic [polyoxyethylene sorbitan monolaurate (Tween 20), octylphenoxy polyethoxy ethanol (Triton X-405), polyvinylpyrrolidone (PVP), polyvinylalchol (PVA, n=500)], and amphoteric [Swanol AM-301] surfactants. However, because an enhancement of fluorescence by the addition of the surfactants had been hardly obtained gained, the surfactant was not used.

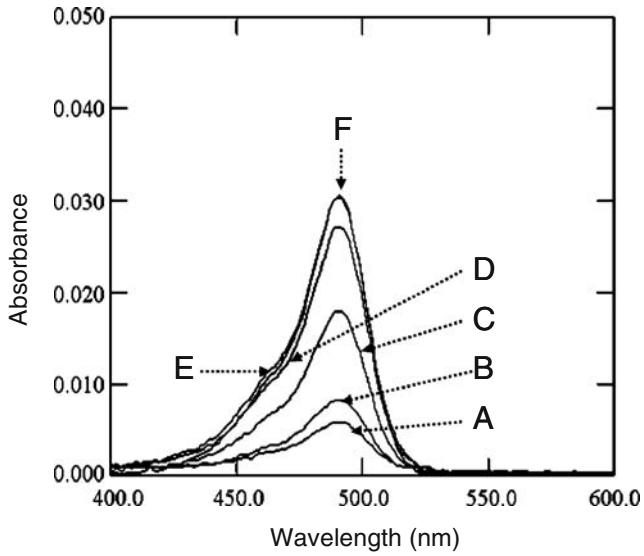

Fig. 2 Absorption spectra of fluorescin and its reaction solution with H_2O_2 as well as fluorescein in the standard procedure solution against the corresponding reagent blank: **a** 4.0×10^{-6}mol L^{-1} fluorescin; **b**–**e** reaction solution of 4.0×10^{-6}mol L^{-1} fluorescin with H_2O_2 (30 ng mL^{-1}, 150 ng mL^{-1}, 300 ng mL^{-1}, 1,500 ng mL^{-1}); and **f** 4.0×10^{-6}mol L^{-1} fluorescein. The conditions employed were 2.0 mL of Tris-HCl buffer solution (pH8.5) and 0.5 mL of 1.0×10^{-4}mol L^{-1} cobalt (II) solution

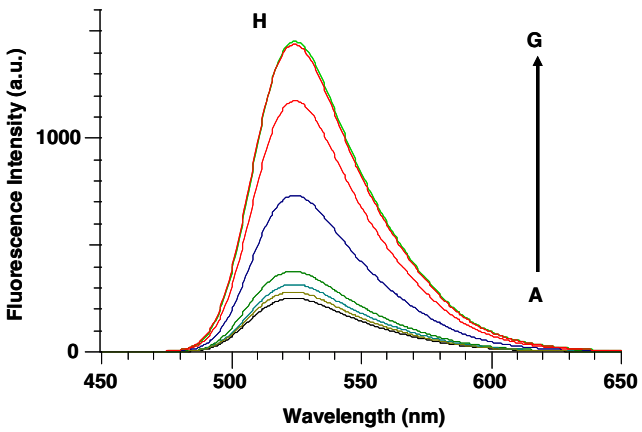

Fig. 3 Fluorescence emission spectra of fluorescin in the absence and presence of H_2O_2 with a standard solution. **a** 4.0×10^{-6}mol L^{-1} fluorescin; **b**–**g** reaction solution of 4.0×10^{-6}mol L^{-1} fluorescin with H_2O_2 (3.0 ng mL^{-1}, 15 ng mL^{-1}, 30 ng mL^{-1}, 150 ng mL^{-1}, 300 ng mL^{-1}, 1,500 ng mL^{-1}); and **h** 4.0×10^{-6}mol L^{-1} fluorescein. The conditions employed were 2.0 mL of Tris-HCl buffer solution (pH8.5) and 0.5 mL of 1.0×10^{-4}mol L^{-1} cobalt (II) solution. The excitation and emission values were selected as 500 nm and 525 nm, respectively. Slit band: Excitation/Emission = 5.0/5.0 nm

The metal ions and catalysts reacted catalytically when small amounts of the metal ions and catalysts coexisted in various oxidation-reduction reactions. Various metal ions and catalysts were tested for the assay of H_2O_2. Cobalt (II) was superior to the various metal ions and catalysts that were tested: zinc (II), iron (II), nickel (II), copper (II), palladium (II), manganese (II), platinum (II), beryllium (II), iron (III), aluminum (III), gold (III), yttrium (III), lanthanum (III), rhodium (III), terbium (III), zirconium (IV), tin (IV), tantalum (V), molybdenum (VI), tungsten (VI), tiron, o-phenanthroline, and potassium chloride. The maximum constant value of RFI was obtained in the fluorescence reaction with 0.5 ml of 1.0×10^{-4} mol L^{-1} Co (II) in the final volume of 10 ml.

The effect of concentration of fluorescin was examined by varying the amounts of the fluorescin solution while maintaining a fixed final concentration of H_2O_2 (ng mL^{-1}). The maximum and almost constant value of RFI was obtained by using 0.4 ml of 1.0×10^{-5} mol L^{-1} fluorescin in the final volume of 10 ml.

The fluorescence development in this reaction system did not occur instantaneously at room temperature. Thus, the effects of an incubation temperature and time were investigated by heating for 10–180 min at 30 °C, 40 °C, and 50 °C. The maximum constant value of RFI was obtained at 40 °C for 30 min, followed by cooling in water for 5 min. The RFI value remained constant for at least 2 h after the solution was cooled to room temperature.

Calibration curve and reproducibility

A relatively stable calibration curve for H_2O_2 was constructed by the standard procedure. A good linear relationship was observed over a wide dynamic range, i.e., 1.5–310 ng mL^{-1} of H_2O_2. The limit of detection, defined as (3.3×SD of blank)/(slope of analytical calibration), was 1.9 ng mL^{-1}. The correlation coefficient was 0.999. Reproducibility tests (n=6) indicated that the relative standard deviations of fluorescence were 2.51% for 3.1 ng mL^{-1}, 2.48% for 30.8 ng mL^{-1}, and 1.31% for 308 ng mL^{-1} of H_2O_2.

Reactivity of fluorescin with various ROS/RNS

We investigated the reactivity of fluorescin with various ROS/RNS. Various oxidants were generated by the following procedure. H_2O_2 (final 10 μmol L^{-1}) was prepared by dilution of a 30% H_2O_2 solution and was corrected by permanganimetry, and a working solution was prepared by the suitable dilution of this stock solution as required. $^1O_2$ (final 10 μmol L^{-1}) was generated by Na_2MoO_4/H_2O_2 system [33]. O_2^- (final 10 μmol L^{-1}) was generated by the reaction of hypoxanthine with xanthine oxidase. Xanthine oxidase was added first. $^{\bullet}OH$ was generated by the reaction of $FeCl_2$ with H_2O_2. $FeCl_2$ (final 10 μmol L^{-1}) solution was added to a standard solution, followed by the addition of H_2O_2 (final 100 μmol L^{-1}) [22]. OCl^- (final 10 μmol L^{-1}) was generated by sodium in NaOCl. NO was generated by 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminoethyl)-3-methyl-1-triazene (NOC7) (final 10 μmol L^{-1}). A standard solution of NOC7 was freshly prepared in a 0.1 mol L^{-1} NaOH solution. $ONOO^-$ (final 10 μmol L^{-1}) was prepared from peroxynitrite solution. $ONOO^-$ levels were determined by using UV at 300 nm. $ROO^{\bullet}$ (final 10 μmol L^{-1}) was generated by 2,2′–azobis(2-amidinopropane)dihydrochloride. All the reactions were performed at 37 for 30 min after the addition of ROS/RNS solutions (each 30 μl) to a standard solution of fluorescin (final 40 μmol L^{-1}, 12 μl), buffer solution (60 μl), and Co (II) solution (final 5 μmol L^{-1}, 15 μl) in each well of a 96-well flat-bottom microtiter plate (Nunc, Denmark), and the fluorescent intensity for each well was measured at 525 nm with excitation at 500 nm (slit width 12 nm). All the fluorescence measurements were performed using a Varioskan fluorescence spectrometer (Thermo Electron). All the results are summarized in Table 1. Fluorescin exhibited fluorescence that was enhanced only upon the reaction with H_2O_2, $^1O_2$, $^{\bullet}OH$, and $ONOO^-$. In addition, the difference in its behavior when cobalt did not coexist is additionally indicated in Table 1. Fluorescin reacted specifically with $ONOO^-$ approximately seven times as compared to other ROS/RNS without Co (II).

Table 1 Fluorescence intensity of fluorescin to various ROS/RNS

ROS/RNS	Fluorescein intensity	
	Co (II)	no Co (II)
Blank	75	31.5
H_2O_2	292	30.5
$^1O_2$	295	34.6
$\cdot O_2^-$	50	26.2
·OH	270.5	43.3
^-OCl	107	30.6
NO	73	30.5
$ONOO^-$	276	231.1
$ROO^{\cdot}$	76	30.6
Autoxidation[a]	85	30

All the reactions were performed at 37 °C for 30 min after the addition of ROS/RNS solutions (final 10 μM, 30 μl) to a standard solution of fluorescin (final 40 μM, 12 μl), buffer solution (60 μl), and Co (II) solution (final 5 μM, 15 μl) in each well of a 96-well flat-bottom microtiter plate (Nunc, Denmark), and the fluorescent intensity for each well was measured at 525 nm with excitation at 500 nm (slit width 12 nm). All the fluorescence measurements were performed using a Varioskan fluorescence spectrometer (Thermo Electron)

[a] Dye solutions were placed under a fluorescent lamp for 2.5 h

Table 2 Effect of foreign substances on determination of H_2O_2

Substances	Added as	Added		Recovery
		ng ml^{-1}	Mole ratio	%
Cu^{2+}	nitrate	6.4×10^3	100	100.0
Mg^{2+}	chloride	2.4×10^3	100	100.0
Zn^{2+}	chloride	6.5×10^3	100	100.0
Ca^{2+}	chloride	4.0×10^3	100	100.0
Fe^{2+}	sulfate	5.6×10^2	10	100.0
Fe^{3+}	alum	0.6×10	1/10	90.0
Mo^{6+}	sodium	9.6×10^2	10	100.0
NaCl	-	5.8×10^3	100	100.0
NaF	-	4.2×10^3	100	91.3
KBr	-	6.0×10^3	50	100.0
KCN	-	6.5×10^2	10	100.0
Na_2HPO_4	-	1.4×10^4	100	100.0
NH_4Cl	-	5.4×10^2	10	100.0
KNO_3	-	1.0×10^4	100	100.0
K_2SO_4	-	1.7×10^4	100	100.0
NH_3	-	1.7×10^3	1000	100.0
Tartaric acid	-	1.5×10^3	100	100.0
Ascorbic acid	-	1.8×10^2	1/10	111.0
Caffeine	-	1.9×10^4	100	100.0
Glucose	-	1.8×10^5	1000	100.0
HSA	-	1.0×10^5	-	100.0
Urea	-	6.0×10^3	100	100.0
Uric acid	-	1.7×10^4	100	100.0
Creatinine	-	1.1×10^4	100	95.1
Alanine	-	8.9×10^4	1000	100.0

H_2O_2 taken, 30.8 ng ml^{-1}; fluorescin, 4.0×10^{-7} M; Co (II), 5.0×10^{-6} M; pH, 8.5; reference, water

Excitation/Emission, 500/524 nm

Interference from foreign substances

For the assessment of the advantages of our method in environmental and clinical assays, the influences of various foreign substances on the determination of H_2O_2 levels were investigated by analyzing synthetic sample solutions that contained 30.8 ng ml^{-1} of H_2O_2. Among the foreign substances, the presence of iron (III) ions and ascorbic acid resulted in a slight increase and decrease in measurement, respectively. The inorganic ions such as copper (II), magnesium (II), zinc (II), calcium (II), iron (II), molybdenum (VI), sodium, chloride, fluoride, potassium, bromide, cyanide, phosphate, ammonium, nitrate, sulfate, and ammonia did not noticeably affect the accuracy of the determination of H_2O_2 levels, even when these ions were present in excessively large amounts as compared to that of H_2O_2. The presence of organic substances such as tartaric acid, caffeine, glucose, HSA, urea, uric acid, creatinine, and alanine caused considerably less interference. The results are summarized in Table 2.

Application

Recovery tests of H_2O_2 in calf serum, human saliva, rain water, and wheat noodles were conducted. Calf serum (Gibco BRL) was treated with deproteinized and centrifuged. Human saliva samples were collected from healthy human volunteers after toothbrushing early in the morning, and the samples were assayed immediately. Rainwater samples were collected in 500 ml polythene bottles using polyethene funnels. The rainwater samples were filtered through a paper filter to remove the small amount of insoluble dry deposition. Wheat noodles were crushed in water, and the suspension was filtered. All the samples were merely diluted 100 times with water. The recoveries of H_2O_2 added to these samples were approximately in the range of 93–108%. This indicates that the proposed method provides accurate results. These results are presented in Table 3.

Conclusion

We have described a simple and highly sensitive fluorophotometric method using fluorescin and cobalt (II) for the determination of H_2O_2 and other ROS /RNS levels over a wide dynamic range. This procedure is based on a redox reaction of H_2O_2 and ROS/RNS with cobalt (II) and fluorescin, one of the three tautometric forms of fluorescein that is a colorless, nonfluorescent lactoid. The proposed method is more sensitive, simple, and reproducible than the previous method [24, 30]. Although further investigations are necessary for the elucidation of this reaction mechanism, the developed procedure is suitable for the analysis of ROS/RNS. In conclusion, this proposed method offers many advantages for the determination of H_2O_2 and ROS/

Table 3 Recovery tests of H_2O_2 added to various samples[a]

Sample	H_2O_2 added (ng ml^{-1})	Found (ng ml^{-1})	Recovery (%)	RSD (%)
saliva	15.4	14.4	93.8	0.98
calf serum	15.4	14.6	94.7	0.69
rain water	15.4	16.6	107.5	0.56
wheat noodles	15.4	15.3	99.3	0.74

fluorescin, 4.0×10^{-7} M; Co (II), 5.0×10^{-6} M; pH, 8.5; reference, water

Excitation/Emission, 500/524 nm

[a] Average of five recovery tests

RNS levels. First, the method is improved with respect to simply procedure and dye in comparison with the former method. Second, the present method contains elements of novelty such as its relative good applicability for measuring hydrogen peroxide in biogenic and environmental samples, such as in calf serum, human saliva and rain water. Finally, this determination method was able to detect stable and strong oxidizing ROS/RNS such as H_2O_2, $^1O_2$, $^{\bullet}OH$, and $ONOO^-$ levels completely.

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