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ORIGINAL PAPER

A Sensitive Resonance Scattering Spectral Assay for the Determination of Trace H_2O_2 Based on the HRP Catalytic Reaction and Nanogold Aggregation

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Abstract Gold nanoparticles in size of 15 nm exhibit a resonance scattering (RS) peak at 580 nm, in pH 6.4 citrate buffer solutions. Horseradish peroxidase (HRP) strongly catalyzed the H_2O_2 oxidation of *o*-phenylenediamine to form an intergradation of cyclohexa-3, 5-diene-1, 2-diylidenediamine and the product of 2, 3-diaminophenazin. Both cause gold nanoparticles aggregations to enhance the RS intensity at 580 nm greatly. Under the optimal conditions, the concentration of H_2O_2 (*C*) in the range of 0.08–2.2 μmol/l was proportional to the enhanced RS intensity at 580 nm ($\Delta I_{580\ nm}$). Its regression equation was $\Delta I_{580\ nm}$=46.8 *C*+ 3.4, with a correlation coefficient of 0.9983 and a detection limit of 0.03 μmol/l H_2O_2. This new RS spectral method was applied to the determination of H_2O_2 in water samples with satisfactory results.

Keywords H_2O_2 · Horseradish peroxidase · *o*-Phenylenediamine · Nanogold · Resonance scattering spectral assay

Introduction

H_2O_2 was widely applied in food, pharmaceutical, clinical, industrial and environmental fields. One of the important factors forming acid rain was owing to the air and rainwater containing H_2O_2. Long-term contact with H_2O_2 would decolor human hair and cause respiratory symptoms. The content of H_2O_2 should be kept under 1.4 mg/m^3 [1] because excessive inhalation of H_2O_2 would lead to poisoning. In addition to, H_2O_2 is the product of some oxidases reaction and the commonly used oxidant in catalytic kinetic analysis. Therefore, the determination of trace H_2O_2 is of great importance. Presently, several methods have been developed for the detection of H_2O_2, including electrochemistry (EC) [2–5], high performance liquid chromatography (HPLC) [6], chemiluminescence (CL) [7, 8], flow-injection analysis (FIA) [9, 10], fluorescence spectrometry (FS) [11], Fourier-transform infrared spectrum (FTIR) [12] and spectrometry [13, 14]. EC method displayed better linear range, but the preparation of electrode was complicated. HPLC method need expensive instrument and the process of sample pre-treatment was time-consuming. Luminol CL method had high sensitivity, but Mn(II) and Co(II) interfered with the assay. FS method can be determined H_2O_2 in the range of 0.05–20 μmol/L, but the used fluorescent reagent was not easy to obtain. A 0.1 μmol/L H_2O_2 can be detected by FTIR, but its operation was complicated and time-consuming. Spectrometry is simple, but sensitivity is lower.

Nanoparticles in liquid phase exhibit novel absorption and scattering spectral characteristics, and become an important research subject in nanochemistry [15]. Gold nanoparticles had easy preparation, high electron density and good biocompatibility, and have been widely applied to nanochemistry, nanostructured material and condensed state physics [16–19]. In immunological analysis, nanogold labeling technique has already been the fourth immune labeling technique [20]. Resonance scattering (RS) or resonance light scattering spectral technique was a sensi-

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tive, rapid and simple analytical technique [21, 22]. RS effect of some nanoparticles was applied in biochemical analysis with good results [23–29]. Recently, RS detection was combined with inorganic catalytic reaction and enzymatic reaction to determine trace metal and enzymatic activity, with high sensitivity and high selectivity [30]. However, it is not found the report about the RS study of horseradish peroxidase catalytic reaction and the nanogold aggregation. In this work, a new highly sensitive and selective, convenient and fast RS spectral method was proposed for the determination of H_2O_2, based on horseradish peroxidase catalytic reaction and nanogold aggregation.

Experimental section

Apparatus A model RF-540 spectrofluorometer (Shimadzu, Japan) was used to record the RS spectrum by synchronous scanning excitation wavelength (λ_{ex}) and emission wavelength (λ_{ex}; $\lambda_{ex}-\lambda_{em}=\Delta\lambda=0$), and to measure the RS intensity with low sensitive file and a longitudinal coordinate scale of 6. A model TU-1901 double beams UV-visible spectrophotometer (Beijing Purkinje General Instrument Limited Company, China), and model H-600 transmission electron microscope (Electronic Stock Limited Company) were used.

Reagents A 1.0% chloroauric acid ($HAuCl_4$) and 2.0 mmol/l *o*-phenylenediamine (OPD; National Pharmaceutical Group Chemical Reagents Company, China) were prepared. A 0.01 mol/l citrate buffer solution (pH 4.8–6.6) was prepared. Hydrogen peroxide (30%, *w*/*v*) was purchased from Shantou Xilong Chemical Factory (Guangdong, China) and its concentration was standardized by titration with potassium permanganate. Horseradish peroxidase (HRP, the RZ was more than 3,300 U/mg) was purchased from Huamei Biotechnology Company, China. All used reagents were of analytical grade and the water was double distilled.

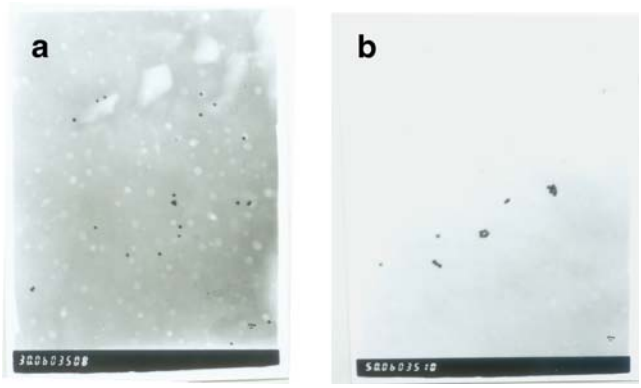


Fig. 1 TEM images of the nanogold systems. **a** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–14.5 μg/ml Au; **b** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–14.5 μg/ml Au–1.63×10^{-6} mol/l H_2O_2

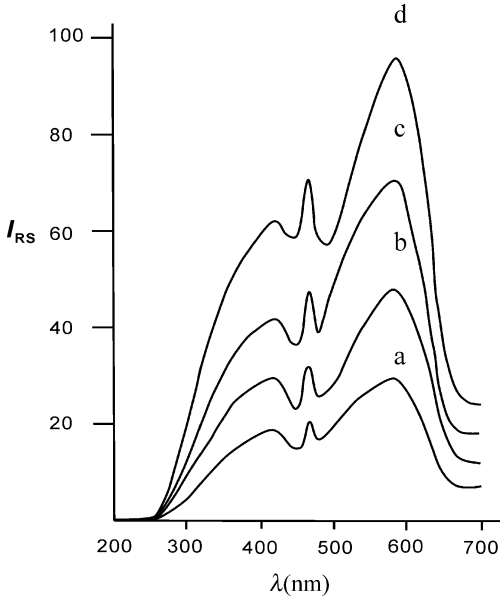


Fig. 2 Resonance scattering spectra. **a** pH 6.4–25 ng/ml HRP-7.5×10^{-5} mol/l OPD-14.5 μg/ml Au; **b** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–14.5 μg/ml Au–4.80×10^{-7} mol/l H_2O_2; **c** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–14.5 μg/ml Au–9.60×10^{-7} mol/l H_2O_2; **d** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–14.5 μg/ml Au–1.44×10^{-6} mol/l H_2O_2

Nanogold in size of 15 nm was prepared as follows [20]. Firstly, 1.0 ml 1.0 % chloroauric acid was added to the 100 ml boiled double distilled water and kept boiling for 5 min. Then 6.0 ml 1.0% trisodium citrate solution was quickly added to the above solution while stirring and kept boiling for 15 min. Finally stopped heating and went on stirring for 15 min. The nanoparticle solution was left to cool to room temperature and was diluted to 100 ml with water. The concentration of the gold nanoparticle was 58.0 μg /ml. It was stored in a refrigerator at 4 °C.

Procedure A 75 μl pH 6.4 citrate buffer solutions, 10 μl 5.0 μg/ml HRP, 75 μl 2.0 mmol/l OPD, a certain quantity of H_2O_2 were successively added to a 5-ml graduated tube, diluted to about 1.0 ml with water and mixed well. After 6 min at 25 °C, a 0.5 ml 58.0 μg/ml nanogold solution was added to the reaction solution. The mixed solutions were diluted to 2.0 ml, and mixed well. Five minutes later, the RS intensity at 580 nm (I) was recorded. The Ib value of the blank solution without H_2O_2 was also measured. The value for $\Delta I_{580\ nm}=I-I_b$ was calculated.

Results and discussion

In pH 6.4 citrate buffer solution, the reaction between OPD and H_2O_2 was very slow. Upon addition of horseradish peroxidase catalyst, formed quickly an intergradation of

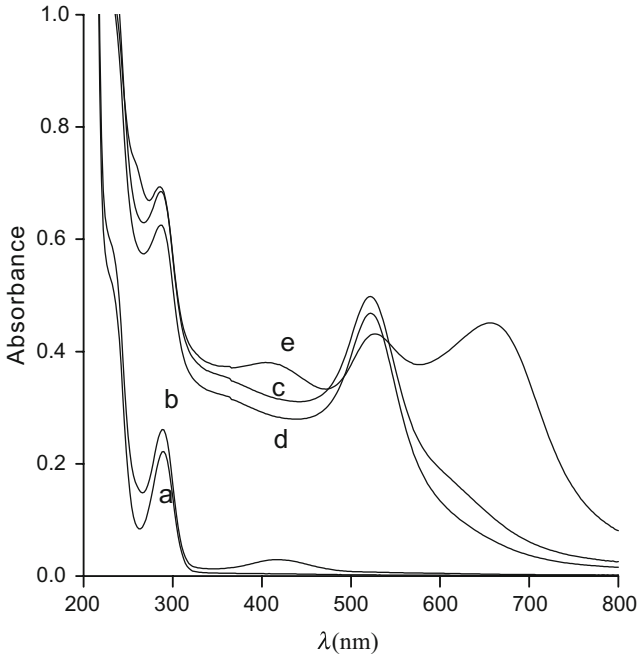
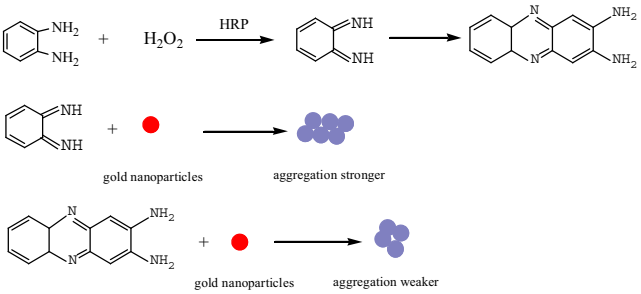


Fig. 3 UV-Vis Absorption spectra. **a** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD; **b** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–1.09×10^{-6} mol/l H_2O_2. **c** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–14.5 μg/ml Au; **d** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–1.09×10^{-6} mol/l H_2O_2–14.5 μg/ml Au; **e** pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–1.63×10^{-6} mol/l H_2O_2–14.5 μg/ml Au

cyclohexa-3, 5-diene-1, 2-diylidenediamine, and a product of 2, 3-diaminophenazin. Upon addition of nanogolds, the aggregations were appeared because there are strong interactions between nanogolds and the oxidization products. The extent of nanogold aggregation increased, and the RS intensity at 580 nm enhanced linearly with the addition of H_2O_2. Based on this principle, a novel enzyme catalytic resonance scattering spectral method for the detection of H_2O_2 was developed.

The change color from red to violet of nanogold aggregation was associated with cationic ion and hydrophobicity [31]. It has been reported that the final oxidization product of OPD was 2, 3-diaminophenazin [32–35]. The order of aggregation effect was cyclohexa-3, 5-diene-1, 2-diylidenediamine, 2,3-diaminophenazin, OPD, because the ionization of cyclohexa-3,5-diene-1,2-diylidenediamine was strongest, liking cationic surfactant. The ionization of OPD and 2, 3-diaminophenazin was weaker. The hydrophobicity of 2, 3-diaminophenazin was stronger that the OPD. Thus, both cyclohexa-3, 5-diene-1, 2-diylidenediamine and 2, 3-diaminophenazin cause the aggregation and change color of nanogolds. The main reaction for the nanogold aggregations was as follows,



Transmission electron microscope (TEM) TEM images showed that gold nanoparticles were in spherical with a mean diameter of about 15 nm in pH 6.4 citrate buffer solution. Upon addition of OPD and HRP, the shape and size did not changed (Fig.1a). Figure 1b indicated that the nanogold particles in the catalytic reaction system were aggregated to big clusters.

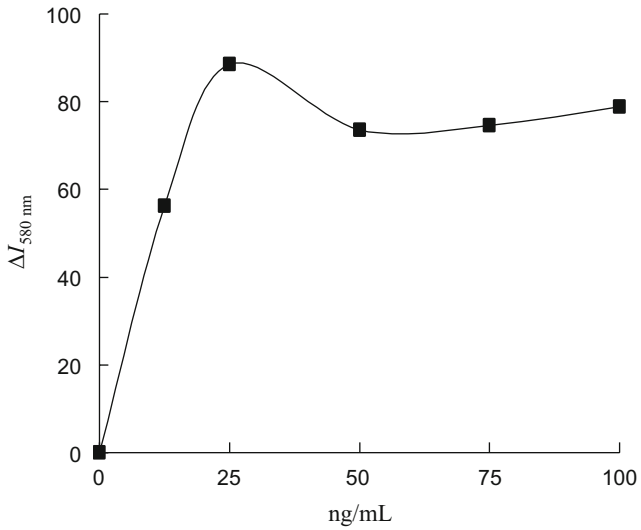


Fig. 4 Effect of HRP concentration. pH 6.4–7.5×10^{-5} mol/l OPD–2.18×10^{-6} mol/l H_2O_2–14.5 μg/ml Au

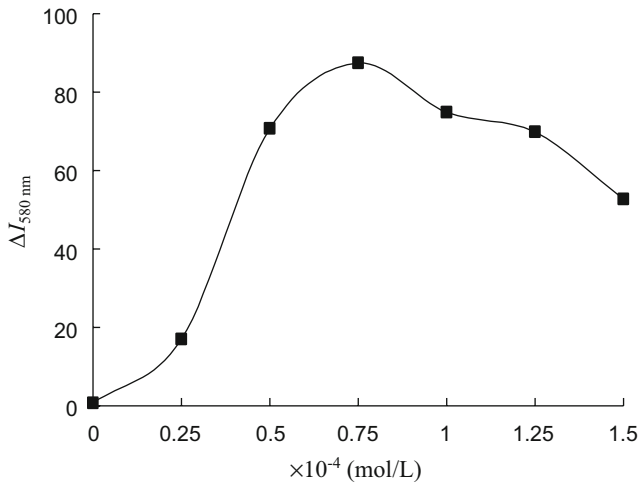


Fig. 5 Effect of OPD concentration. pH 6.4–25 ng/ml HRP–2.18×10^{-6} mol/l H_2O_2–14.5 μg/ml Au

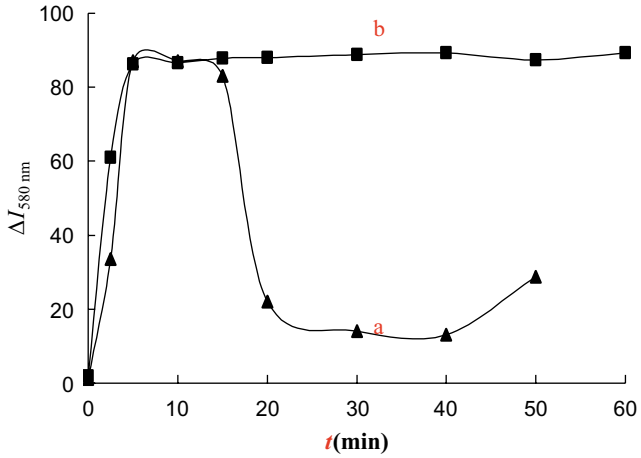


Fig. 6 Effect of reaction time. pH 6.4–25 ng/ml HRP–7.5×10^{-5} mol/l OPD–2.18×10^{-6} mol/l H_2O_2–14.5 μg/ml Au. **a** Enzyme catalytic reaction time; **b** The aggregation reaction time

Resonance scattering spectra and absorption spectra Light scattering is a commonly optical phenomenon, and is the interaction between the incident photon and the interface electron on the particle such as nanoparticle and supermolecule etc. It was known that there are some electrons on the nanoparticle surface, in which electrons located in the ground state or excited state. The energy between the ground state and the excitation state was called as excited energy. Changing the incident photon energy by scanning the excited wavelength, when the incident photon energy is same or close at the excited energy of surface electrons, in which the incident photon resonate with the electron, that cause the scattering signal enhanced greatly. This optical phenomenon was called as RS effect or resonance light scattering effect. This synchronous scattering spectrum, including RS effect, was called as RS spectrum. Figure 2 indicated that the catalytic aggregation system exhibited three synchronous scattering peaks at 420, 470 and 580. The strongest peak was at 580 nm. The synchronous scattering study of inorganic nanoparticles indicate that three factors including the light source of the apparatus, free molecular absorption, and the RS effect of particles caused synchronous scattering spectral peaks [30]. The strongest emission of the apparatus was at 470 nm. Thus, the nanogold system appeared a synchronous scattering peak at 470 nm. The absorption spectra of the system (Fig. 3) indicated that OPD appeared a molecular absorption peak at 290 nm, and the oxidation product of OPD has a weak molecular absorption peak at 420 nm. Nanogold in size of 15 nm exhibited a surface plasmon resonance absorption peak at 515 nm, the aggregation of nanogold has a wide surface plasmon resonance absorption peak at 650 nm. For the catalytic aggregation system, the molecular absorption of citrate, HRP, OPD and H_2O_2 was rather weak in the visible light region, and their effect on synchronous scattering spectra can be neglected. Therefore the synchronous scattering peaks at 420 and 580 nm were caused by the nanogold RS effect, and the both were RS peak. With addition of H_2O_2 increasing, the RS intensity at 580 nm enhanced linearly. Thus, a wavelength of 580 nm was selected.

Effect of pH value The effect of pH 4.8–6.6 and buffer solution volume on the $\Delta I_{580\ nm}$ was investigated respectively. Results showed that the $\Delta I_{580\ nm}$ value reached its maximum when the pH value was 6.4. Thus, a pH 6.4 was selected for the system. A 75 μl pH 6.4 citrate buffer solutions, giving max $\Delta I_{580\ nm}$ value, was used.

Effect of HRP concentration HRP was a catalyst for the reaction. The costs of assay can be cut by using small amount of HRP. The effect of the concentration of HRP on the $\Delta I_{580\ nm}$ value was examined. As seen in Fig. 4, the $\Delta I_{580\ nm}$ reached its maximum when the concentration of HRP was 25 ng/ml. Thus, a 25 ng/ml HRP was chosen for assay. The use amount of HRP was less.

Effect of OPD concentration Effect of three substrates including *o*-phenylenediamine (OPD), *m*-phenylenediamine and *p*-phenylenediamine on the HRP catalytic aggregation system was examined respectively. OPD had higher sensitive and was chosen for use. Influence of OPD concentration on the $\Delta I_{580\ nm}$ was showed in Fig. 5. When OPD concentration was 0.75×10^{-4} mol/l, the $\Delta I_{580\ nm}$ value of the system reached its maximum. Thus, a concentration of 0.75×10−4 mol/l OPD was selected for use.

Table 1 Comparison of some assays for H_2O_2

Methods	Linear range (μmol/l)	DL (μmol/l)	Remarks	Reference
Electrochemistry	5–400	0.9	Low sensitivity	[2]
High performance liquid chromatography		5.9l	Expensive and complicated	[6]
Chemiluminescence	1.0–1,000		Complicated	[7]
Flow injection analysis	0.04–5.0		Simple and sensitive	[10]
Ultraviolet-visible spectrophotometry	1.5–1,500		Low sensitivity	[13]
	1.3–41	0.6	Simple, speed	[15]
Enzyme catalytic RS assay	0.08–2.2	0.03	Sensitive, simple and cheap	This report

Table 2 Influence of coexisting substance (CS) (1.0 μmol/l H_2O_2)

Coexisting Substance	Tolerance [CS]/[H_2O_2]	Relative error (%)	Coexisting Substance	Tolerance [CS]/[H_2O_2]	Relative error (%)
Cu^{2+}	80	+1.4	Mn^{2+}	50	−1.7
Co^{2+}	100	−1.2	Al^{3+}	100	+4.7
Fe^{2+}	100	−4.5	Pb^{2+}	100	+3.5
Zn^{2+}	100	−2.7	Glu	50	−2.2
Ni^{2+}	25	−3.2	L-Lysine	25	+2.8
Cr^{3+}	10	+3.2	L-proline	100	+1.5
Hg^{2+}	10	+2.4	Phenylalanine	100	−1.4
Ca^{2+}	80	−3.1	EDTA	100	+2.3
Mg^{2+}	25	−2.3	BSA	50	+2.8

Effect of gold nanoparticles concentration The effect of 15 nm nanogold, 30 nm nanogld, 50 nm nanogold, 20 nm nanosilver, and 15 nm nanoplatinum on the RS intensity of HRP catalytic-nanoparticle aggregation system was considered respectively. The 15 nm nanogold aggregation system gives high sensitivity, and chosen for use. The effect of the nanogold concentration on the $\Delta I_{580\ nm}$ value was tested. The results showed that when the nanogold concentration was up to 12 μg/ml, the $\Delta I_{580\ nm}$ value increased linearly. When its concentration was more than 13 μg/ml, the $\Delta I_{580\ nm}$ value was biggest and stable. A 14.5 μg/ml gold nanoparticles was selected for use.

Effect of reaction time The effect of HRP catalytic and the nanogold aggregation reaction times on the $\Delta I_{580\ nm}$ was considered respectively. Results indicated that the $\Delta I_{580\ nm}$ value reached its maximum when the catalytic reaction time was in the range of 5–15 min (Fig. 6a), and the nanogold aggregation reaction time was in the range of 5–60 min (Fig. 6b). The RS quenching of the Fig. 6a and the change color effect of gold nanoparticle were associated with the HRP reaction products. It was known that the intergradation of cyclohexa-3, 5-diene-1, 2-diylidenediamine was unstable and easy ionization. The content of the intergradation was bigger in the catalytic reaction time of 5–15 min. Thus, a platform as in the Fig. 6a was occurred. A 6 min HRP reaction time and 5 min nanogold aggregation time were selected.

Effect of reaction temperature The effect of reaction temperature (20–70 °C) on the $\Delta I_{580\ nm}$ was considered. Results showed that the $\Delta I_{580\ nm}$ value reached its maximum when the reaction temperature was in the range of 20–30 °C. A reaction temperature of 25 °C was chosen.

Working curve Under the optimal conditions, the $\Delta I_{580\ nm}$ for different H_2O_2 concentration (C) was obtained. The $\Delta I_{580\ nm}$ is proportional to the C in the range of 0.08–2.2 μmol/l. The regression equation is $\Delta I_{580\ nm}=46.8C+3.4$, with the correlation coefficient of 0.9983 and a detection limit (DL) of 0.03 μg/ml H_2O_2. Compared with previously reported assays [2–10, 13, 15] (Table 1), this HRP catalytic RS method is sensitive and selective, rapid and cheap.

Influence of coexisting substances The influence of coexistence substances on the determination of 1.0 μmol/l H_2O_2 was investigated according to the procedure, with a relative error of ±5.0 %. The results are summarized in Table 2. It can be seen that the coexistence substances did not interfered with the determination of H_2O_2, which demonstrated the method has good selectivity.

Table 3 Analytical results of H_2O_2 (n=5)

Sample	H_2O_2 content (μg/ml)	Added H_2O_2 (μg)	Found H_2O_2 (μg)	Recovery (%)	Ref. results[14] (μg/ml)
1	0.0486±0.0011	0.0370	0.0392	106	0.0474
2	0.0362±0.0018	0.0370	0.0362	98.1	0.0380
3	0.121±0.0019	0.0370	0.0368	99.5	0.115
4	0.0200±0.0016	0.0370	0.0371	100	0.0220
5	0.0324±0.0015	0.0370	0.0347	93.7	0.0313
6	0.0228±0.0013	0.0370	0.0399	108	0.0235
7	0.0212±0.0011	0.0370	0.0367	99.1	0.0203
8	0.0548±0.0021	0.0370	0.0381	103	0.0539
9	0.0992±0.0030	0.0370	0.0373	101	0.0897
10	0.101±0.0033	0.0370	0.0376	102	0.116

Analysis of samples The rain water samples were taken using a glass sampling bottle and funnel, and kept at 4 °C. The water samples were filtrated by 150 nm quartz filtration film, and were assayed by this HRP catalytic RS method, with a recovery of 93.7~106.0%. The results are summarized in Table 3, and were agreement with that of the reference results [14].

Conclusions

A resonance scattering spectral method is developed for the determination of trace H_2O_2, based on HRP catalytic reaction and the nanogold aggregation. Its linear range is from 0.08 to 2.2 μmol/l, with a detection limit of 0.03 μmol/l H_2O_2. The method was applied to analysis of H_2O_2 in water samples with satisfactory results. Results demonstrated that the aggregation of nanogold caused mainly by the intergradation of cyclohexa-3, 5-diene-1, 2-diylidenediamine.

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