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A new red-region substrate, tetra-substituted amino aluminium phthalocyanine, for the fluorimetric determination of H_2O_2 catalyzed by mimetic peroxidases

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A new red-region fluorogenic substrate, tetra-substituted amino aluminium phthalocyanine, was developed for the selective determination of H_2O_2 based on the catalytic effect of mimetic peroxidases, *viz*., hemin or iron tetrasulfonatophthalocyanine (FeTSPc). Under the optimum conditions, the linearity of the calibration graph for the determination of H_2O_2 with hemin (or FeTSPc) as the catalyst was in the range from 0.0 to 3.0×10^{-7} mol L^{-1} (or from 0.0 to 2.0×10^{-6} mol L^{-1}). The detection limits were 3.7×10^{-9} and 4.9×10^{-9} mol L^{-1} H_2O_2, respectively. The relative standard deviation ($n = 7$) was within 1.5% in the middle of the linear range. The peroxidase activity of the mimetic enzymes hemin and FeTSPc, the effects of some experimental conditions and the influence of foreign substances were investigated. With this substrate, 0.0–7.5×10^{-8} mol L^{-1} hemin and 0.0–2.0×10^{-6} mol L^{-1} FeTSPc can be determined with an accuracy and precision of about 1.3%. The potential application of the reagent was tested by the determination of H_2O_2 in rainwater.

1 Introduction

Horseradish peroxidase (HRP)-catalyzed fluorogenic reactions have found wide application in the determination of H_2O_2.[1–6] HRP, however, is expensive and its solution is not sufficiently stable. Therefore, the use of stable mimetic peroxidases as a substitute for HRP in fluorescence reactions has increased markedly in recent years.[7–14] Hemin and iron–tetrasulfonatophthalocyanine (FeTSPc) have been found to exhibit very significant catalytic activity for the peroxidation reaction between some fluorogenic substrates and H_2O_2[11–14] and to be capable of being used as an efficient substitute for HRP.

It is well known that the choice of substrates for HRP and HRP mimetics has an important influence on the sensitivity of the method. To date, a wide variety of fluorogenic substrates are known for HRP and mimetic peroxidases. Of the substrates proposed, *p*-hydroxyphenylacetic acid (*p*-HPA), homovanillic acid (HVA), *p*-hydroxyphenylpropionic acid (*p*-HPPA) and tyramine have been extensively studied and are regarded as the best choice for the determination of H_2O_2.[15–19] However, the oxidation products of these compounds have their excitation and fluorescence maxima in a relatively short wavelength region, *viz*., 310–320 and 400–420 nm, respectively. In many instances, this results in a strong background fluorescence and scattered light signal. In addition, the substrates, such as *p*-HPA and HVA, are also expensive and not easily available. Hence, a new trend in the area is the application of dyes with fluorescence bands in the red or near-infrared region, for example, the cyanine and phthalocyanine compounds.

In this work, a red-region fluorescent dye, 4, 4′, 4″, 4‴-tetraamino aluminium phthalocyanine (denoted as TAAlPc, which displays an excitation maximum at 610 nm and an emission maximum at 678 nm in strongly acidic medium, see Fig. 1), was employed for the selective determination of H_2O_2 catalyzed by mimetic peroxidases. Because most luminescent molecules have their absorption and fluorescence emission in the region below 600 nm,[20] the background fluorescence and the scattered light of the samples can be minimized by use of TAAlPc as the substrate and a high sensitivity and selectivity of the method is achieved. Moreover, the fluorescent dye is easily synthesized in large amounts, making this method suitable for practical application. Under the catalytic action of hemin or FeTSPc, trace amounts of H_2O_2 rapidly react with TAAlPc, thus quenching the fluorescence of TAAlPc. The peroxidase activity of the mimetic enzymes hemin and FeTSPc was also investigated and our experimental results showed that the peroxidase activity of hemin is higher than that of FeTSPc. Application of hemin- and FeTSPc-catalyzed fluorescence quenching of TAAlPc to the determination of hemin and FeTSPc led to a highly sensitive system, providing detection limits of 5.8×10^{-10} mol L^{-1} hemin and 1.7×10^{-9} mol L^{-1} FeTSPc. The potential of TAAlPc as a new red-region substrate for mimetic peroxidases was further proved by the determination of H_2O_2 in rainwater samples.

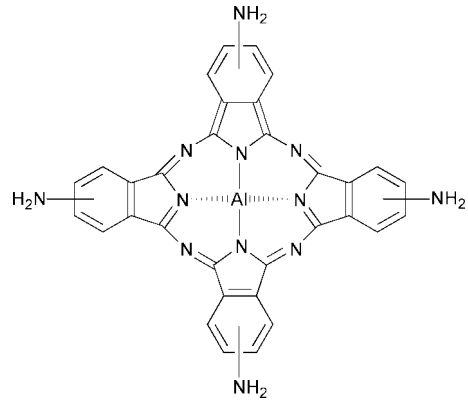

Fig. 1 Molecular structure of TAAlPc.

2 Experimental

2.1 Reagents

A hemin stock solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving 32.6 mg of hemin in 50.0 mL of 0.01 mol L^{-1} NaOH. This solution is stable when refrigerated. FeTSPc was synthesized and purified according to Weber and Busch,[21] and a stock standard aqueous solution of 0.001 mol L^{-1} was prepared; it was stable at room temperature for several months. A H_2O_2 stock solution (3%, v/v) was prepared by diluting the commercial reagent (30%, v/v, Shanghai Taopu Chemical Factory); its concentration was standardized by titration with standard $KMnO_4$ solution. A stock solution of TAAlPc (1.0×10^{-3} mol L^{-1}) was prepared by dissolving an appropriate amount of TAAlPc, which was synthesized and purified according to the ref. 22, in redistilled *N*,*N*-dimethylformamide. A 0.05 mol L^{-1} Tris–HCl buffer solution was prepared by mixing 125 mL of 0.1 mol L^{-1} Tris and 36.8 mL of 0.1 mol L^{-1} HCl and diluting the mixture to 250 mL. A 3.0 mol L^{-1} HCl solution was used. All chemicals were of analytical-reagent grade. Distilled, deionized water was used throughout.

2.2 Apparatus

A Hitachi 650-10S fluorescence spectrophotometer equipped with a plotter unit and a 1 cm quartz cell was used to record the spectra and make the fluorescence measurements. A Beckman DU-7400 ultraviolet–visible spectrophotometer was used to record absorption spectra. A digital pH-meter (Xiamen Analytical Instrument Factory) was employed to make the pH measurements.

2.3 Measurement of the catalytic activity of hemin and FeTSPc with TAAlPc as a substrate

The peroxidase activities of hemin and FeTSPc were measured by the initial rate method.

2.3.1 Peroxidase activity of hemin. To a set of 10 mL calibrated tubes, 1.0 mL of 0.05 mol L^{-1} Tris–HCl buffer solution (pH 8.5), 0.05 mL of 1.0×10^{-5} mol L^{-1} hemin solution, 0.8 mL of 0.01 mol L^{-1} H_2O_2 solution and various volumes of TAAlPc were added. The mixtures were quickly adjusted with water to a fixed volume and allowed to stand at room temperature for 1 min. The relative fluorescence intensities of the solutions were measured at 678 nm with an excitation wavelength of 610 nm after 0.50 mL of 3.0 mol L^{-1} HCl solution had been added and mixed. The data were treated by the Michaelis–Menten equation.

2.3.2 The peroxidase activity of FeTSPc. To a set of 10 mL calibrated tubes, 1.0 mL of 3.0 mol L^{-1} HCl, 0.05 mL of 1.0×10^{-4} mol L^{-1} FeTSPc solution, 0.1 mL of 0.01 mol L^{-1} H_2O_2 solution and various volumes of TAAlPc were added. The mixtures were quickly diluted with water to volume and allowed to stand at room temperature for 1 min. The relative fluorescence intensities were measured at 678 nm with excitation at 610 nm. The data were treated by the Michaelis–Menten equation.

2.4 Analytical procedure for the determination of H_2O_2

2.4.1 Determination of H_2O_2 using hemin as the catalyst. To a set of 10 mL tubes containing 1.0 mL of 0.05 mol L^{-1} Tris–HCl (pH 8.5), 0.20 mL of 1.0×10^{-4} mol L^{-1} hemin and 0.05 mL of 1.0×10^{-4} mol L^{-1} TAAlPc, various volumes of H_2O_2 standard solutions were added and diluted to a fixed volume with water. The mixtures were allowed to stand for 8 min at room temperature; the fluorescence intensities were then measured at 678 nm with an excitation wavelength of 610 nm after 0.50 mL of 3.0 mol L^{-1} HCl had been added and mixed.

2.4.2 Determination of H_2O_2 using FeTSPc as the catalyst. To a set of 10 mL tubes containing 1.0 mL of 3.0 mol L^{-1} HCl, 0.20 mL of 1.0×10^{-4} mol L^{-1} FeTSPc and 0.15 mL of 1.0×10^{-4} mol L^{-1} TAAlPc, various volumes of H_2O_2 standard solutions were added and diluted to 10.0 mL with water. The mixtures were allowed to stand for 8 min at room temperature. Measurements of the relative fluorescence intensities of the solutions were then made at 678 nm with excitation at 610 nm.

3 Results and discussion

3.1 Spectral characteristics

Fig. 2 is the electronic absorption spectra of TAAlPc in the presence of H_2O_2 in hydrochloric acid medium. It is obvious that TAAlPc has two absorption bands, a Soret band and a Q band, with the maximum wavelength located at about 347 and 673 nm, respectively. When different concentrations of H_2O_2 were mixed with TAAlPc, no significant wavelength shift occurred for the peaks, however; the absorption peaks of both the Soret band and the Q band obviously decreased with increasing amounts of H_2O_2. The excitation and emission spectra of the hemin–TAAlPc–H_2O_2 and hemin–TAAlPc systems in acidic media are shown in Fig. 3(A) and (B). It can be seen that the fluorescence emission peak of TAAlPc is located at 678 nm and two excitation peaks occur, one in the short-wavelength region (Soret band), and the other in the long wavelength region (Q band). When excited with the Soret band, the secondary scattered light would cause interference. In addition, the determination was subject to the interference resulting from the fluorescence of the matrix. Therefore, 610 nm was chosen as the excitation wavelength. As can be seen from Fig. 3, the fluorescence spectra of the hemin–TAAlPc–H_2O_2 system are similar in shape to those of the hemin–TAAlPc system, that is, the fluorescence excitation and emission wavelengths remained at 610 and 678 nm, respectively. However, the fluorescence intensity of the hemin–TAAlPc system is dramatically quenched when H_2O_2 is present. In order to explore the reason for the fluorescence quenching of

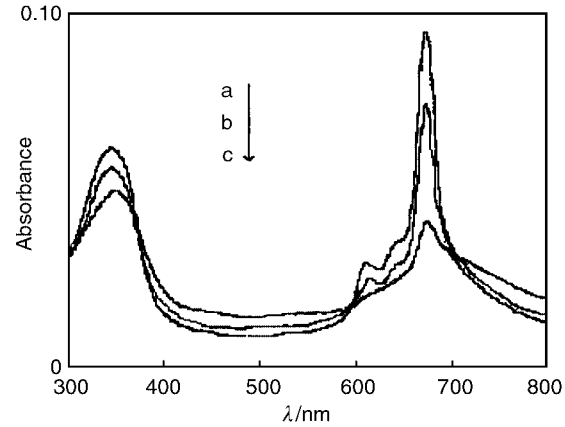

Fig. 2 Absorption spectra of TAAlPc. TAAlPc, 1.0×10^{-6} mol L^{-1}; hemin, 2.0×10^{-6} mol L^{-1}; concentration of H_2O_2 (mol L^{-1}): a, 0; b, 1.0×10^{-7}; c, 5.0×10^{-7}.

TAAlPc, two other phthalocyanines, tetra-substituted sulfonated aluminium phthalocyanine (AlS_4Pc) and tetra-substituted carboxyl aluminium phthalocyanine (AlC_4Pc), were also reacted with H_2O_2 under the catalytic action of hemin. The results indicated that, among the three phthalocyanine compounds tested, only the fluorescence of TAAlPc could be greatly quenched by H_2O_2 in the presence of hemin. This suggested that the fluorescence quenching of TAAlPc might be due to the oxidation of the amino groups of TAAlPc by H_2O_2 under the catalytic action of hemin.

When FeTSPc is used as a catalyst, the absorption and fluorescence spectra of the FeTSPc–TAAlPc–H_2O_2 system are similar to those of the hemin–TAAlPc–H_2O_2 system.

3.2 Comparison of the catalytic characteristics of hemin and FeTSPc with TAAlPc as a substrate

As mentioned above, the catalytic activity of hemin and FeTSPc in the fluorescence quenching reaction of TAAlPc and H_2O_2 was studied by the initial rate method with a steady-state assumption[23] where the concentration of H_2O_2 was saturated in the tested system. The Michaelis–Menten constant K_m and the maximum rate V_{max} were obtained from a Lineweaver–Burk plot (1/*V versus* 1/[substrate]).[24] Since $V_{max} = K_{cat}[E_0]$, where $[E_0]$ is the initial concentration of enzyme, the transformation constant K_{cat}, which represents the enzymatic catalytic activity, could be obtained. The greater the value of K_{cat}, the greater will be the enzymatic activity. A comparison of the catalytic parameters for hemin and FeTSPc is given in Table 1. It can be seen that the catalytic activity of hemin is much higher than that of FeTSPc when taking TAAlPc as a substrate. This fact further confirmed that the catalytic activity of mimetic enzymes largely depended on the substrate used.

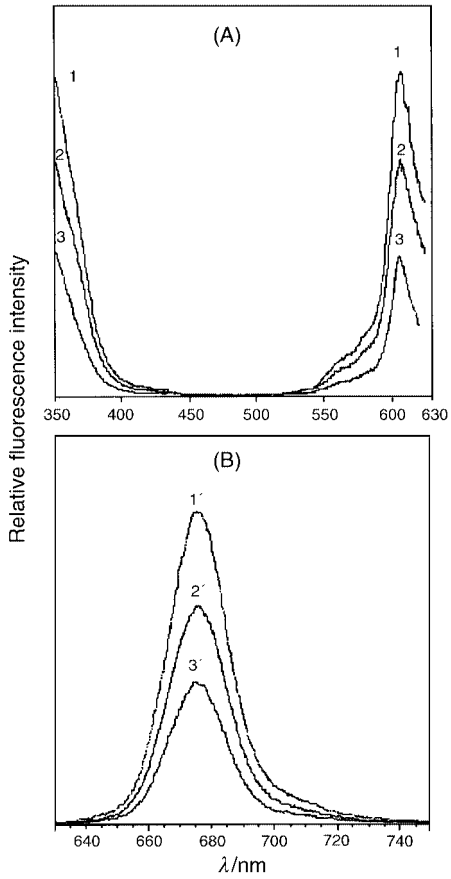

Fig. 3 Fluorescence excitation spectra (A) (λ_{em} = 678 nm) and emission spectra (B) (λ_{ex} = 610 nm) of hemin–TAAlPc (1, 1′) and hemin–TAAlPc–H_2O_2 (2, 2′; 3, 3′) systems. Hemin, 2.0×10^{-6} mol L^{-1}; TAAlPc, 1.0×10^{-6} mol L^{-1}; H_2O_2: (2, 2′), 1.0×10^{-7} mol L^{-1}; (3, 3′), 2.0×10^{-7} mol L^{-1}.

3.3 Optimization of experimental parameters

3.3.1 Effect of pH and buffer. The effect of pH on the hemin (or FeTSPc)-catalyzed reaction was examined. Generally, the catalytic reaction and the final fluorescence development were carried out at different optimum pH values.[15] Our experiments indicated that the optimum pH for the hemin-catalyzed reaction was around 8.5. Among the four types of buffer solution tested, that is, Tris–HCl, hexamethylenetetraamine (HT)–HCl, glycine–NaOH and NH_3–NH_4Cl, Tris–HCl buffer solution provided the maximum quenching. Since the ionic strength may affect the hemin-catalyzed reaction, the optimum amount of Tris–HCl buffer solution was also studied. The results showed that the optimum amount of Tris–HCl lay in the range 0.80–1.20 mL. Therefore, 1.0 mL of 0.05 mol L^{-1} Tris–HCl buffer solution was used throughout. However, the pH for the final fluorescence development should be controlled in a strongly acidic medium. In our experiments, 0.5 mL of 3.0 mol L^{-1} hydrochloric acid was added to the system. As for the FeTSPc–TAAlPc system, pH control is different from that of the hemin–TAAlPc system. The FeTSPc-catalyzed reaction of TAAlPc with H_2O_2 occurs best at 0.6 mol L^{-1} HCl, which is also the optimum acidity for the final fluorescence development. This would significantly simplify the analytical procedure.

3.3.2 Reaction time and temperature. The influence of reaction time on the fluorescence quenching was investigated with the use of hemin as the catalyst (Fig. 4). From Fig. 4, it can be seen that the background fluorescence (without H_2O_2) also decreased slowly with time due to the oxygen dissolved in the water. In the presence of H_2O_2, the oxidation of TAAlPc occurred rapidly; the fluorescence difference between the TAAlPc–hemin–H_2O_2 and TAAlPc–hemin systems was max-

Table 1 Catalytic parameters when using hemin or FeTSPc as the catalyst and TAAlPc as the substrate

Mimetic enzyme	$K_m/10^{-6}$ mol L^{-1}	$V_{max}/10^{-8}$ mol L^{-1} s^{-1}	$[E_0]/10^{-7}$ mol L^{-1}	$K_{cat}/10^{-2}$ s^{-1}
Hemin	0.79	1.27	0.5	25.4
FeTSPc	1.01	0.344	5.0	0.69

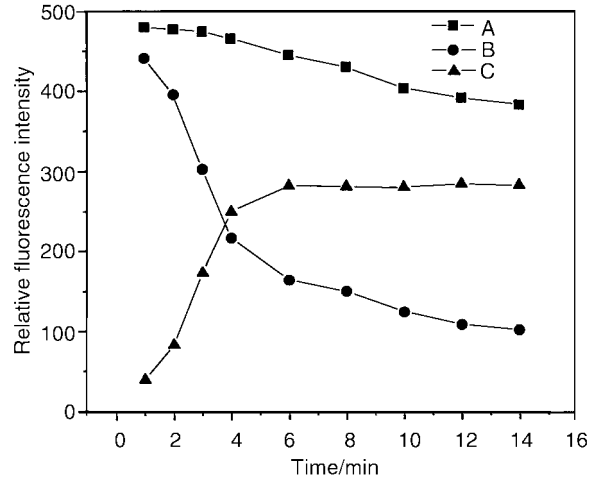

Fig. 4 Kinetic curves for the fluorescence quenching of TAAlPc in the presence and absence of H_2O_2. A: Hemin–TAAlPc; B: hemin–TAAlPc–H_2O_2; C: $\Delta F = F_B - F_C$. TAAlPc, 1.0×10^{-6} mol L^{-1}; hemin, 2.0×10^{-6} mol L^{-1}; H_2O_2, 2.0×10^{-7} mol L^{-1}.

imum and constant after reacting for 6 min. The same was true for the kinetic profile of the reaction of TAAlPc with H_2O_2 in the presence of FeTSPc. Therefore, the equilibrium method was chosen, and a reaction time of 8 min was adopted.

The catalytic reaction was also temperature-dependent. Experiments showed that the net fluorescence quenching value was maximum and constant in the temperature range 10–40 °C. Therefore, room temperature was used to carry out the catalytic oxidation reaction and for the measurement of fluorescence intensity.

3.3.3 Optimum amounts of catalyst and TAAlPc for the determination of H_2O_2. Our experimental results indicated that the maximum fluorescence quenching was reached when the concentration of hemin (or FeTSPc) was in the range from 1.6×10^{-6} to 2.6×10^{-6} mol L^{-1}. In this work, a concentration of 2.0×10^{-6} mol L^{-1} was chosen for both hemin and FeTSPc. The influence of TAAlPc concentration on its fluorescence quenching was investigated. It was found that, the lower the concentration of TAAlPc, the greater the sensitivity of the method, but at the expense of the linear range. Taking both the sensitivity and the linear range into account, a final concentration of 5.0×10^{-7} and 1.5×10^{-6} mol L^{-1}TAAlPc was chosen for the hemin–TAAlPc and FeTSPc–TAAlPc systems, respectively.

3.3.4 Optimum amounts of H_2O_2 and TAAlPc for the determination of hemin and FeTSPc. Tests showed that suitable concentrations of H_2O_2 and TAAlPc in the final solution were 8.0×10^{-4} and 1.0×10^{-6} mol L^{-1}, respectively, for the determination of hemin, whereas for the determination of FeTSPc, suitable concentrations of H_2O_2 and TAAlPc in the final solution were in the range from 8.0×10^{-5} to 1.5×10^{-4} and from 1.0×10^{-6} to 2.0×10^{-6} mol L^{-1}, respectively. Therefore, 0.1 mL of 1.0×10^{-2} mol L^{-1} H_2O_2 and 0.15 mL of 1.0×10^{-6} mol L^{-1} TAAlPc was selected.

3.4 Features of the analytical method

The possibility of using the proposed substrate for the determination of H_2O_2 and mimetic peroxidases was studied. The calibration graphs for the determination of H_2O_2 and mimetic peroxidases were constructed under the optimum conditions described above. The results are listed in Table 2. The detection limits of H_2O_2 and mimetic peroxidases were given by the equation LOD = KS_0/S, where K is a numerical factor chosen according to the confidence level desired, S_0 is the standard deviation of the blank measurements and S is the slope of the calibration graph. Here, a value of 3 for K was used. It is shown in Table 2 that the graphs obtained from the hemin–TAAlPc system have lower detection limits for H_2O_2 and hemin than those obtained from the FeTSPc–TAAlPc system. Comparison of the performance of the proposed method with that of other methods for the determination of H_2O_2 is summarized in Table 3. It can be seen that the hemin (or FeTSPc)–TAAlPc–H_2O_2 system shows a comparable linear range and sensitivity.

3.5 Effects of foreign substances

The above results demonstrate that the proposed method is applicable to the determination of H_2O_2. For the assessment of its usefulness in environmental assays, the effects of certain foreign substances generally present in environmental water samples were examined in the determination of 2.0×10^{-7} mol L^{-1} H_2O_2 with the hemin–TAAlPc–H_2O_2 system; the results are summarized in Table 4. It can be seen from Table 4 that inorganic ions such as Na^+, Cl^-, K^+, NO_3^-, SO_4^{2-}, PO_4^{3-} and Ca^{2+} did not noticeably affect the accuracy of H_2O_2 determination. A positive interference was observed from Fe^{3+} ion present at 2.5 times the concentration of H_2O_2. A possible reason for this interference might be that Fe^{3+} ion acts as an additional catalyst in the hemin-catalyzed system and thus causes a positive error. A further study indicated that adding a suitable amount of EDTA could mask the interference from Fe^{3+} ion because of the formation of a stable complex of Fe^{3+} ion with EDTA. Our experimental results indicated that the permissible concentration of Fe^{3+} ion would be 1.0×10^{-5} mol L^{-1} when the EDTA concentration was 2.0×10^{-5} mol L^{-1} in the hemin–TAAlPc–H_2O_2 system. However, when the

Table 3 Comparison of the analytical characteristics of some mimetic enzyme-catalyzed fluorimetric methods for the determination of H_2O_2

Catalyst[a]	Substrate[b]	$\lambda_{ex}/\lambda_{em}$/nm	Linear range/10^{-7} mol L^{-1}	LOD/10^{-8} mol L^{-1}	Ref.
Hemin	Thiamine	373/440	0.0–50	3.0	11
FeTSPc	*p*-HPPA	324/409	0.0–36	1.3	13
Mn-TMPyP	HVA	316/424	1.3–24	—	7
Mn-TPPS4	HVA	424/318	0.0–25	8.5	25
Hemin	DCM–OPA	360/454	0.2–10	0.76	26
Hemin	MDHQ	344/404	0.2–10	0.76	26
Hemin	*p*-HPPA	320/406	0.08–0.8 1–10	0.46	26
Hemin	TAAlPc	610/678	0.0–3.0	0.37	This work
FeTSPc	TAAlPc	610/678	0.0–20	0.49	This work

[a] TMPyP : tetrakis (*N*-methylpyridiniumyl) porphyrin. $TPPS_4$: tetrakis(sulfophenyl)porphyrin. [b] DCM–OPA : *N*,N¹-dicyanomethyl-*o* phenylenediamine. MDHQ : 3-methyl-3,4-dihydroquinoxalin-2(1H)-one.

Table 4 Tolerance of foreign substances in the determination of 2.0×10^{-7} mol L^{-1} H_2O_2

Foreign substance	Tolerance (molar ratio)	Relative error (%)	Foreign substance	Tolerance (molar ratio)	Relative error (%)
Na^+	10 000	+1.7	Cu^{2+}	250	+3.4
NO_3^-	10 000	+4.7	Pb^{2+}	250	+5.1
S^{2-}	10 000	−3.8	Sn^{2+}	250	−6.1
Cl^-	10 000	−1.1	Mg^{2+}	250	+2.7
NH_4^+	5 000	+1.6	Zn^{2+}	250	+1.7
PO_4^{3-}	5 000	+3.4	EDTA	100	−3.4
SO_4^{2-}	5 000	+5.1	Al^{3+}	100	+3.8
K^+	5 000	+2.5	Hg^{2+}	50	+4.3
SO_3^{2-}	2 000	−2.5	Mn^{2+}	10	+2.5
Ca^{2+}	2 000	+3.0	Ni^{2+}	10	+2.2
Cd^{2+}	1 000	+2.5	NO_2^-	10	−3.4
Co^{2+}	250	+2.6	Fe^{3+}	2.5	+4.3

Table 2 Analytical characteristics for the determination of H_2O_2 and mimetic peroxidases

System	Analyte	Linear range/mol L^{-1}	Detection limit/mol L^{-1}	Correlation coefficient
Hemin–TAAlPc–H_2O_2	H_2O_2	$0.0–3.0 \times 10^{-7}$	3.7×10^{-9}	0.992
	Hemin	$0.0–7.5 \times 10^{-8}$	5.8×10^{-10}	0.998
FeTSPc-TAAlPc-H_2O_2	H_2O_2	$0.0–2.0 \times 10^{-6}$	4.9×10^{-9}	0.996
	FeTSPc	$0.0–2.0 \times 10^{-6}$	1.7×10^{-9}	0.994

system is applied to the determination of H_2O_2 in rainwater, addition of EDTA is not necessary due to the small amount of sample used and the trace levels of metal ions in the samples.

3.6 Determination of H_2O_2 in rainwater samples

The proposed method was used to determine H_2O_2 in rainwater samples. The samples were collected and filtered before analysis. The accuracy of the method was investigated by performing recovery tests on standard additions to real samples. The results obtained are presented in Table 5. The recovery experiments gave satisfactory results.

4 Conclusion

The results obtained in this work reveal that: (1) TAAlPc can serve as an efficient red-region fluorescent substrate for mimetic peroxidases. (2) Using the hemin (or FeTSPc)–H_2O_2–TAAlPc catalytic system, the quantification of H_2O_2 and mimetic peroxidases can be performed, and the proposed method can be applied to determine trace amounts of H_2O_2 in rainwater. (3) The catalytic activity of hemin is far higher than that of FeTSPc with TAAlPc used as a substrate, *i.e.*, using the hemin–H_2O_2–TAAlPc system for the determination of H_2O_2 and hemin provides a higher analytical sensitivity. (4) Owing to the acidity adopted, the analytical procedure of using the FeTSPc-catalyzed system is more simple than that of using the hemin-catalyzed system. (5) The spectral characteristics of TAAlPc enable the interference from background fluorescence and scattered light to be greatly reduced.

Table 5 Determination of H_2O_2 in rainwater samples (n = 3)

Sample	Concentration of H_2O_2/ 10^{-8} mol L^{-1}	H_2O_2 added/ 10^{-8} mol L^{-1}	Recovery (%)
Rainwater 1	5.86	4.0	95.7
Rainwater 2	8.54	4.0	99.0
Rainwater 3	13.0	5.0	94.0

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