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FIA–near-infrared spectrofluorimetric trace determination of hydrogen peroxide using tricarchlorobocyanine dye (Cy.7.Cl) and horseradish peroxidase (HRP)

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Abstract

A new kind of near-infrared fluorescence agent, tricarbochlorocyanine dye (Cy.7.Cl), had been synthesized in house and used for near-infrared spectrofluorimetric determination of hydrogen peroxide (H_2O_2) by flow injection analysis (FIA) for the first time. The oxidation reaction of Cy.7.Cl with H_2O_2 occurred under the catalysis of horseradish peroxidase (HRP) and it was studied in detail. The possible reaction mechanism was discussed. Under optimal experimental conditions, fluorescence from Cy.7.Cl displayed excitation and emission maxima (ex/em) at 780 and 800 nm, respectively. The two linear working ranges were 1.86×10^{-7} to 4.11×10^{-7} mol L^{-1} and 4.11×10^{-7} to 7.19×10^{-6} mol L^{-1}, respectively. The detection limit was 5.58×10^{-8} mol L^{-1} of H_2O_2. The effect of interferences was studied. The proposed method was successfully applied to the determination of hydrogen peroxide in rainwater, serum and plant samples.

Keywords: FIA–near-infrared spectrofluorimetry; Tricarbochlorocyanine dye; Hydrogen peroxide; HRP

1. Introduction

Hydrogen peroxide widely exists in biological systems and environment. It is the natural product in the oxidation metabolic process of biology being harmful to organism [1–3]. It is a source of toxic oxygen that produces $HO^{\bullet}$ an even more toxic species [4], which can lead to disease and senescence of body. Normally, the concentration of H_2O_2 produced in the organism is kept at a low level because it can be eliminated by the defensive system of body. However, it will cause damage to body when the concentration of H_2O_2 reaches 0.5 mmol L^{-1}. Therefore, the trace determination of H_2O_2 is very important in environmental analysis, biochemical analysis and for clinic diagnostic. In addition, many biological substances produce H_2O_2 in biochemical reactions catalyzed by various enzymes, so they can be determined indirectly by the determination of hydrogen peroxide. In recent years, various methods for the determination of H_2O_2 have been proposed, such as spectrofluorimetry [5–9], spectrophotometry [10,11], electrochemistry [12–16], chemiluminescence [17–21], and so on. The horseradish peroxidase (HRP)-catalyzed reaction is one of the most widely used enzymatic reactions [22–25]. The characteristics of the enzyme have been systematically studied with H_2O_2 as oxidizing agent and in the presence of various substances as fluorogenic substrates. Based on these catalytic reactions, various highly sensitive spectrofluorimetric methods for determination of H_2O_2 have been developed [6,26–28]. However, the oxidation products of these fluorescence agents had excitation and emission maxima in a relatively short wavelength region of 300–420 nm. When H_2O_2 was determined in this region, there were interferences from background of the complex matrix, which reduced the selectivity of methods. If detection was carried on in the near-IR region of the spectrum (750–1000 nm), background interferences from biological components was much lower

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than those in the visible region. So, researchers have been more interested in the use of near-IR spectrofluorimetry to detect biological compounds such as DNA [29], protein [30], amino acid [31], and so on. But its application to the determination of H_2O_2 has not been proposed up till now. In our studies, we found that traces of H_2O_2 could rapidly react with Cy.7.Cl under the catalysis of HRP, which decreased the fluorescence of Cy.7.Cl. In this paper, Cy.7.Cl has been synthesized and used as a new kind of near-infrared fluorescence agent for the determination of H_2O_2 with high sensitivity. The best advantage of proposed method is the less interference from biological background because the measurement is carried out at the near-IR excitation and emission wavelengths ($\lambda_{ex/em}$ = 780/800 nm). In addition, the solubility in water is better for Cy.7.Cl is an inner salt, which avoids the use of organic solvents in the determination. Moreover, quantification of hydrogen peroxide is rather difficult because of its rapid decomposition (5% h^{-1}), so the sample determination must be done as quickly as possible in order to obtain precise and reliable analytical data. Flow injection analysis (FIA) can achieve real-time, online, rapid and automated analysis, which can decrease the error caused by detection time. Therefore, FIA–near-infrared spectroflurimetric method is proposed in this paper, which is successfully applied to the determination of H_2O_2 in rainwater, serum and plant samples. To our knowledge, this is the first report on using a near-infrared fluorescence agent for the determination of hydrogen peroxide.

2. Experiment

2.1. Apparatus and reagents

2.1.1. Apparatus

The fluorescence spectra and intensity were measured on Cary Eclipse spectrofluorimeter with a xenon lamp and 18 μl quartz flow-through cell (Varian, Australia). The flow injection apparatus equipped with an eight-channel actuated injection valve and two peristaltic pumps (FIA-3100, Beijing Wantuo, China) was used. All pH measurements were made with a pH-3C digital pH-meter (Shanghai Lei Ci Device Works, China). The IR spectra were recorded on PE-983 IR spectrometer (Aqueous Solution, Perkin-Elmer, Norwalk, CT, USA). Elemental analysis was performed on PE-240 CHN elementary analytical meter (Perkin-Elmer, Norwalk, CT, USA). The ^{1}H NMR spectra were recorded on FX-300Q Nuclear Magnetic Resonance Spectrometer (DMSO as solvent, JEOL, Japan). The absorbance was recorded on UV-265 spectrophotometer (Shimadzu, Japan).

2.1.2. Reagents

2,3,3-Trimethylindolenine (Beijing Chengyu Specialty Chemical Co. Ltd.), 1,3-propane sultone (Wuhan Feng Fan Surface Engineering Co. Ltd.), phosphorus oxychloride and cyclohexanone were the main materials used in synthesis. The 2.5×10^{-3} mol L^{-1} Cy.7.Cl (fluorescence agent, synthesized in house) stock solution was prepared by dissolving an appropriate amount of fluorescence agent in ethanol and diluted to 5.0×10^{-5} mol L^{-1} with doubly distilled water. The 1.0×10^{-2} g L^{-1} HRP (Sigma, activity > 300 unit/mg enzyme) stock solution was diluted to 5.0×10^{-4} g L^{-1} and stored in the refrigerator. A stock solution of H_2O_2 (0.1027 mol L^{-1}) was standardized by titration with potassium permanganate. Sodium citrate–HCl buffer solution (pH = 4.90, 0.10 mol L^{-1}) was used as the carrier. The 1.0×10^{-3} mol L^{-1} NaN_3 (Beijing Chemical Reagents Company) was prepared in water. All chemicals used were of analytical or high-reagent grade. Doubly distilled water was used throughout.

2.2. Synthesis of Cy.7.Cl

2.2.1. 2,3,3-Trimethyl-1-(3-sulfopropyl)-3H-indolium, inner salt (a) [31]

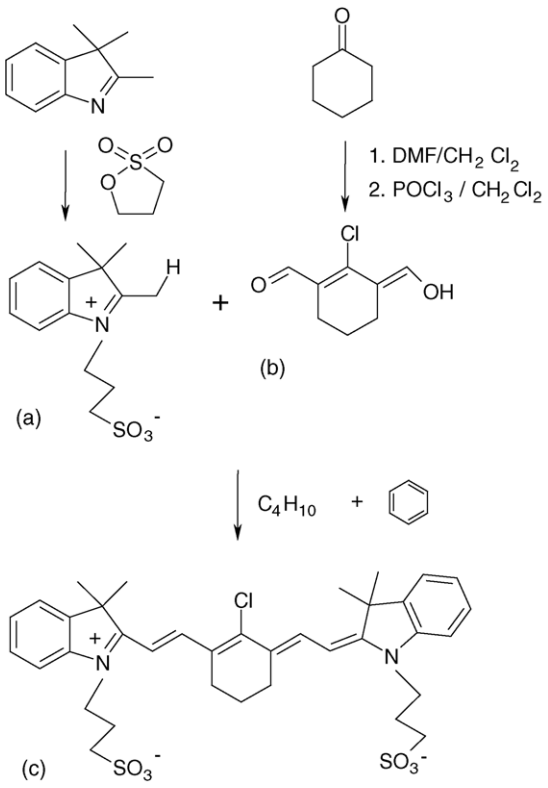
Toluene (50 mL), 2,3,3-trimethylindolenine (10 mL, 0.062 mol), and 1,3-propane sultone (8.2 mL, 0.094 mol) were mixed and heated under reflux for 18 h. Then the reaction mixture was cooled to room temperature and filtered. The precipitate was washed with acetone (3 × 10 mL). The pink crystals (15.2 g, yield 86.8%) were crystallized from MeOH–Et_2O solution. Melting point was measured (126–128 °C). Elemental analysis (%) calculated for $C_{14}H_{20}NO_3S$ (found): C 59.6 (59.3), H 7.1 (7.0), N 5.0 (5.2).

2.2.2. 2-Chloro-1-formyl-3-hydroxymethylenecyclohexene (b) [32]

Forty milliliters of dimethylformamide previously mixed with 40 mL of methylene chloride was chilled in an ice bath, then 37 mL of phosphorus oxychloride dissolved in 35 mL of methylene chloride was added dropwise with stirring, followed by 10 g of cyclohexanone. The solution was refluxed for 3 h, cooled, poured onto 200 g of ice, and allowed to stand overnight. The yellow solid was crystallized from a small volume of acetone cooled with dry ice, to give 14.5 g (82.4%) with the melting point of 130–131 °C. Elemental analysis (%) calculated for $C_8H_9ClO_2$ (found): C 55.7 (55.4), H 5.3 (5.3), Cl 20.5 (20.4).

2.2.3. Cy.7.Cl (c) [33]

Quaternary salt (a) (1.2 g, 4 mmol) and 0.35 g (2 mmol) of bisaldehyde (b) were dissolved in 150 mL of a mixture of 1-butanol and benzene (7:3) in a flask equipped with a Dean–Stark trap. The mixture was heated in reflux with constant stirring and the water formed was collected in the trap. After 3 h, the reaction was cooled to room temperature, and the solvents were removed under vacuum. The residue was washed with ether to give 1.4 g of pure product with yield of 99%. Elemental analysis (%) calculated for $C_{36}H_{44}O_6N_2S_2Cl$ (found): C 61.8 (61.6), H 6.3 (6.5), N 4.0 (4.1), Cl 5.1 (5.0). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.27 (d, 4H), 7.62 (d, 2H), 7.54 (d, 2H), 7.42 (t, 2H), 7.27 (t, 2H),

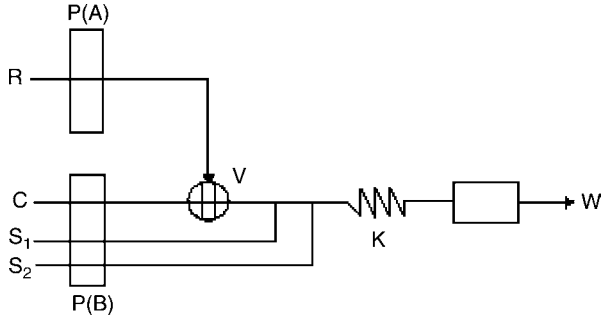


Scheme 1. The synthetic route of fluorescence agent.

6.53 (d, 2H), 4.30 (t, 4H), 2.75 (t, 4H), 2.56 (br t, 4H), 2.03 (m, 4H), 1.83 (m, 4H), 1.67 (s, 12H). The synthetic route was shown in Scheme 1.

2.3. Flow injection analysis assembly

Preliminary tests were carried out with the aid of different flow assemblies to select the optimal manifold configuration. The assembly in Scheme 2 was selected for it produced the best compromise between peak height and shape. In the assembly, the solution of fluorescence agent was injected through the valve, which was called reverse-injection. Reverse-injection could economize the fluorescence agent and the peak shape was better than normal-injection. The operation program was shown in Table 1.



Scheme 2. A schematic diagram of instrumental set-up. P(A) and P(B): pumps A and B; V: valve; K: single bead string reactor (SBSR, length = 90 cm, i.d. = 0.80 mm); D: detector; W: waster; R: Cy.7.Cl (5.0×10^{-5} mol L^{-1}); C: carrier (pH = 4.90, 0.10 mol L^{-1} of sodium citrate–HCl buffer solution); S_1: H_2O_2 or water; S_2: HRP (5.0×10^{-4} g L^{-1}); sampling time: 15 s; injection time: 13 s; stopped-flow time: 80 s; pumps rotate speed: 30 rpm.

Table 1
The operation program in FIA

Process[a]	Valve location	Time (s)	Rotate speed (rpm)	
			P(A)	P(B)
(1) Sampling	S	15	30	30
(2) Injection	I	13	0	30
(3) Stopped-flow	I	80	0	0
(4) Flow into detector	I	10	0	30

S: sampling; I: injection. Valve location (S or I) was switched automatically.
[a] The circulations were five times.

2.4. Determination of H_2O_2

Actuating the peristaltic pump A (P(A), Scheme 2), Cy.7.Cl (R) was sampled into the injection loop when the valve located sampling. Then Cy.7.Cl sampled in the loop was injected into the single bead string reactor (K, Scheme 2) by the carrier stream when the valve located injection. Other reagents were injected into K directly with the actuated pump B. H_2O_2 was mixed with Cy. 7.Cl and HRP. Cy.7.Cl was oxidized by H_2O_2 (reaction catalyzed by HRP) in the reactor for 80 s (stopped-flow time). Then, the mixture passed into the detector cell of the spectrofluorimeter, where the fluorescence intensity was measured at 800 nm with excitation at 780 nm. The reagent blank was measured using water (S_1). The relative fluorescence intensity (ΔF) was inversely proportional to the amounts of H_2O_2. The experimental parameters were set as follows: injection volume, 200 μl; the reaction tube length, 90 cm (0.80 mm i.d.); the excitation and emission slits were 5 and 10 nm, respectively. Other parameters were shown in Table 1.

2.5. Sample treatment

Three different rainwater samples in the same day were filtered and diluted 10-fold to perform the determination in the same day.

Three different normal adult serum samples were diluted 100-fold with doubly distilled water, in which 1.0×10^{-5} mol L^{-1} of NaN_3 was added to restrain catalase (CAT) activity. Then the samples were determined.

Hydrogen peroxide in plant leaves was extracted referring to Ferguson method [34]. Two grams of cole leaves and 10.00 mL of cool acetone were mixed and pounded into a paste. Then the mixture was centrifuged at 4000 rpm. The supernatant was collected and two aliquots of 1.00 mL were selected. The first one was decolored with 4.00 mL of extractant (CCl_4:$CHCl_3$ = 3:1) and 5.00 mL of doubly distilled water. After equilibrated and centrifuged, the supernatant of H_2O_2 extract was obtained and diluted 10-fold

for determination. The other without decoloration was added into 5.00 mL of water, then diluted 10-fold for determination.

3. Results and discussion

3.1. Discussion of reaction mechanism

In order to determine the optimal working wavelength, the spectral characteristics of Cy.7.Cl and its oxidized product by H_2O_2 were studied. The results (Fig. 1) showed that Cy.7.Cl had strong fluorescence in the near-IR region of the spectrum since it contained larger conjugated system. Under the optimal experimental conditions, Cy.7.Cl formed fluorescence at 780/800 nm (Fig. 1a). Its fluorescence intensity decreased after Cy.7.Cl reacted with H_2O_2 (Fig. 1b) due to the rupture of the conjugated double bond in the structure of Cy.7.Cl by oxidation. Since there was a linear relationship between the relative fluorescence decreasing (ΔF) and the amounts of H_2O_2, the sensitive quantitative determination of H_2O_2 could be done. Under the catalysis of HRP the reaction was notably improved (Fig. 1c). Such conditions for a faster reaction are suitable for automation (FIA).

In order to discuss the mechanism of the reaction, we studied the absorption spectra characteristics of Cy.7.Cl and its oxidation in the presence of H_2O_2. The results (Fig. 2) showed that the absorbance of Cy.7.Cl at 774 nm decreased and a strong absorption at 278 nm appeared. In addition, the IR spectrum of the mixture of products showed the characteristic carbonil peak (C=O) at 1639 cm^{-1}. A suggested mechanism for this reaction was shown in Scheme 3.

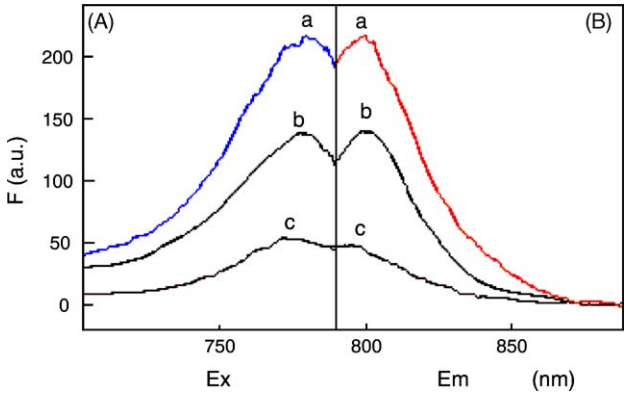


Fig. 1. Excitation (A) and emission (B) spectra of the oxidation reaction and reagent blank. (a) Cy.7.Cl + HRP + buffer solution; (b) Cy.7.Cl + H_2O_2 + buffer solution; (c) Cy.7.Cl + HRP + H_2O_2 + buffer solution; C (Cy. 7.Cl): 5.0×10^{-5} mol L^{-1}; C (HRP): 5.0×10^{-4} g L^{-1}; C (H_2O_2): 3.5×10^{-6} mol L^{-1}; buffer solution: sodium citrate–HCl (pH = 4.90, 0.10 mol L^{-1}).

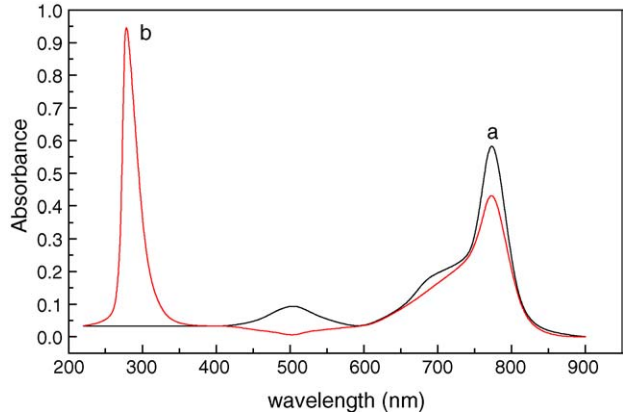


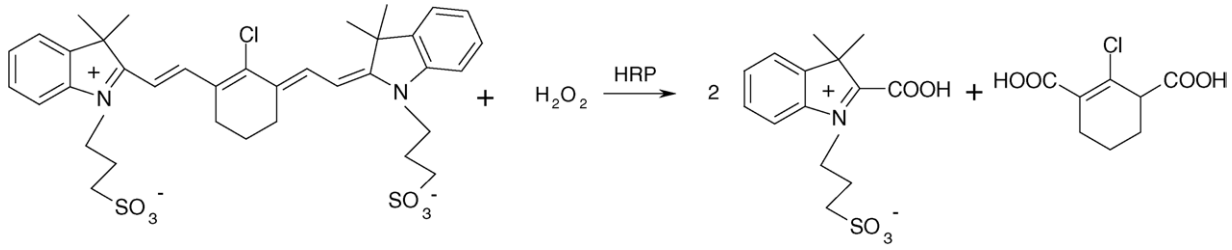
Fig. 2. Absorption spectra of the reaction system before (a) and after (b) oxidation. (a) Cy.7.Cl + HRP + buffer solution; (b) Cy. 7.Cl + HRP + H_2O_2 + buffer solution; C (Cy. 7.Cl): 5.0×10^{-5} mol L^{-1}; C (HRP): 5.0×10^{-4} g L^{-1}; C (H_2O_2): 3.5×10^{-6} mol L^{-1}; buffer solution: sodium citrate–HCl (pH = 4.90, 0.10 mol L^{-1}), 2.00 mL.

3.2. Optimization of manifold parameters

The variables studied for optimization of the manifold parameters were sampling, injection and stopped-flow time, injection volume and flow rate. The reagents used in these experiments were as follows: Cy.7.Cl (5.0×10^{-5} mol L^{-1}), HRP (5.0×10^{-4} g L^{-1}), H_2O_2 (3.5×10^{-6} mol L^{-1}) and sodium citrate–HCl (pH = 4.90, 0.10 mol L^{-1}) buffer solution as the carrier.

3.2.1. Effect of sampling, injection and stopped-flow time

Sampling and injection time had an important effect on the sensitivity and peak shape. The results showed that ΔF was highest and the peak shape was better when sampling and injection time were 15 and 13 s, respectively. Stopped-flow time which has been applied to the determination of H_2O_2 [35] was an important factor to the completeness degree of the reaction, influencing accuracy of the analytical results. The



Scheme 3. The mechanism of the reaction.

experiment showed that when the stopped-flow time reached 80 s, the reaction had been completed, therefore, a stopped-flow time of 80 s was chosen. These parameters allowed a sample throughput of 30 samples h^{-1}.

3.2.2. Effect of flow rate

The reactor was a piece of PTFE tube (90 cm long and 0.80 mm i.d.). The flow rate was an important factor to sample throughput. In this experiment, the flow rate was adjusted by changing rotate speed of the pumps. The results showed that setting the rotating speed at 30 rpm and flow rates at 1.60, 2.40, 2.20 and 2.20 mL min^{-1}, respectively, for the carrier solution, Cy.7.Cl, H_2O_2 and HRP, the ΔF was maximized with a stable baseline and good peak shape. So, the rotating speed was chosen as 30 rpm and the flow rates of carrier, Cy.7.Cl, H_2O_2 and HRP were 1.60, 2.40, 2.20 and 2.20 mL min^{-1}, respectively.

3.2.3. Effect of injection volume

The injection volume of Cy.7.Cl solution was varied between 50 and 300 μl. The results showed that the relative fluorescence intensity increased with the increase of injection volumes up to 200 μl, then the ΔF magnitude flatted for concentrations up to 300 μl. As bigger injection volume meant lower sample throughput, 200 μl was chosen in order to guarantee the sensitivity and high sample throughput synchronously. Chosen manifold parameters were shown in Table 1.

3.3. Effect of pH

The pH had a great effect on the oxidation capacity of H_2O_2, influencing the sensitivity of the determination. The results indicated that the optimal pH range was from 4.50 to 5.40 (Fig. 3). Therefore, a pH of 4.90 was selected. Among NH_4Ac–HCl, HAc–NaAc and sodium citrate–HCl buffer solution systems, the proposed method was more sensitive using sodium citrate–HCl buffer solution (pH = 4.90, 0.1 mol L^{-1}) which was chosen as the carrier.

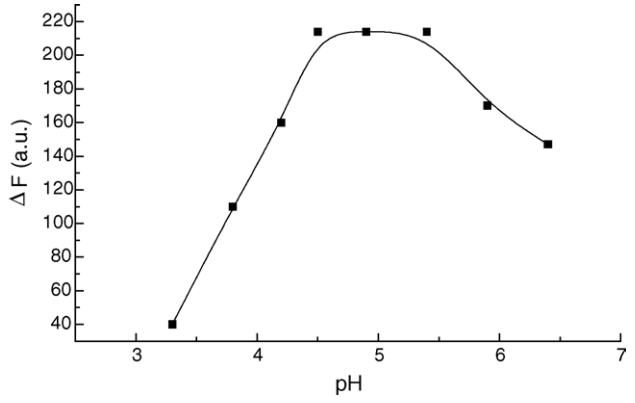


Fig. 3. Effect of pH. C (Cy. 7.Cl): 5.0×10^{-5} mol L^{-1}; C (HRP): 5.0×10^{-4} g L^{-1}; C (H_2O_2): 3.5×10^{-6} mol L^{-1}; sampling time: 15 s; injection time: 13 s; stopped-flow time: 80 s; pumps rotate speed: 30 rpm.

3.4. Effect of Cy.7.Cl concentration

The ΔF increased as the fluorescence agent concentration was increased up to 4.0×10^{-5} mol L^{-1}, then ΔF kept stable for higher concentrations. Therefore, the Cy.7.Cl concentration was 5.0×10^{-5} mol L^{-1}.

3.5. Effect of HRP concentration

Horseradish peroxidase enzyme had an obvious catalysis on the experiment system. The results showed that the ΔF was maximized for the HRP concentration range between 3.0×10^{-4} and 1.0×10^{-3} g L^{-1}. Hence, 5.0×10^{-4} g L^{-1} HRP was chosen.

3.6. Study of potential interferences

Twenty-six potential interferents were studied individually to investigate their effects on the determination of 3.50×10^{-6} g L^{-1} H_2O_2. An error of ±5.0% in the relative fluorescence intensity was considered tolerable. No interferences were found for (tolerable ration in mol): sucrose, table sugar (4000); lactin, tryptophan, DL-tyrosine, L-phenylalanine (3500); thymine, cytosine, K^+, Cl^- (3000); adenine, guanine, Mg^{2+}, SO_4^{2-} (2500); Na^+, Zn^{2+}, Ca^{2+}, Al^{3+} (2000); DNA, RNA, HAS (1500); lactose, BSA, Fe^{3+}, I^-, NO_3^- (1000). The experimental results indicated that the proposed method had proper selectivity to be applied for biological fluids.

3.7. Analytical characteristics

Under the optimal experimental conditions, there were two distinct linear working ranges in function of the H_2O_2 concentration in the range of 1.86–4.11($\times 10^{-7}$) mol L^{-1} and 4.11–71.9($\times 10^{-7}$) mol L^{-1} with the correlation coefficient (r) of 0.9996 and 0.9992, respectively. The corresponding regression equation were $\Delta F = 102.24C(\times 10^{-6}\ \text{mol L}^{-1}) - 5.52$ and $\Delta F = 23.30C(\times 10^{-6}\ \text{mol L}^{-1}) + 28.78$, respectively. The detection limit was 5.58×10^{-8} mol L^{-1} as defined by the IUPAC. The relative standard deviation was 1.3% obtained from a series of 11 standards each containing 1.0×10^{-6} mol L^{-1} H_2O_2. The analytical characteristics of the proposed method were compared with other procedures described in the literature (Table 2). Compared to the 10 first references, the proposed method had lower limit of detection and wider linear range. Although the detection limits described in Table 2, 11*–13*, were lower than the proposed method, the wavelengths used for determination were in the UV–vis region, so the background fluorescence may cripple their application in biological samples. In addition, most of these methods could not be automated. Since fluorogenic reactions are too slow.

Table 2
Comparison of characteristics of the proposed method with those published previously for H_2O_2 determination

Method	Linear range ($\times10^{-7}$ mol L^{-1})	Detection limit ($\times10^{-8}$ mol L^{-1})	Ref.
(1)Glassy carbon electrode covered with polyion complex membrane	17–100	50	[13]
(2)Immobilization of HRP to nano-Au monolayer modified chitosan-entrapped carbon paste electrode	122–24300	630	[14]
(3)Electrochemiluminescence with self-containing reactant biochips	20–5000	200	[15]
(4)Microperoxidase-11 biosensor	200–24000	80	[36]
(5)Amperometric biosensor	5.0–540	12	[37]
(6)FIA–amperometry	10–1000	29	[38]
(7)Sol–gel HRP biosensor by chemiluminescence	1000–30000	67000	[19]
(8) Chemiluminescence flow biosensor	8–1000	28	[20]
(9) Spectrofluorometric method with Ce(III)	–	10	[8]
(10) HRP-like catalyst copper 2-hydroxy-1-naphthaldehyde-2-aminothiazole [Cu^{II}-$(HNATS)_2$] with spectrophotometric method	56–1100	170	[11]
(11*) Flow-injection method based on HRP-catalysed fluorescent reaction of H_2O_2 with *p*-hydroxyphenylpropionic acid as fluorogenic substrate ($\lambda_{ex/em}$ = 318/405 nm)	0.04–0.8	0.2	[9]
(12*) Based on tetra-substituted amino aluminum phthalocyanine as a new red-region substrate ($\lambda_{ex/em}$ = 610/678 nm)	0.0–2.0	0.14	[7]
(13 *) Fluorometric scopoletin–horseradish peroxide method ($\lambda_{ex/em}$ = 365/495 nm)	0.036–0.446	0.11	[6]
The proposed method	1.86–71.9	5.58	

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

Sample	Sample content ($\times10^{-6}$ mol L^{-1})	Added in addition ($\times10^{-6}$ mol L^{-1})	Found total ($\times10^{-6}$ mol L^{-1})	Recovery (%)
(1)	5.95 ± 0.02	6.00	11.82 ± 0.03	98.2
(2)	3.83 ± 0.03	4.00	7.91 ± 0.02	102.0
(3)	4.25 ± 0.01	4.00	8.16 ± 0.02	97.8

Table 4
Determination of H_2O_2 in adult serum and recovery (n = 5)

Sample	Sample content ($\times10^{-7}$ mol L^{-1})	Added in addition ($\times10^{-7}$ mol L^{-1})	Found total ($\times10^{-7}$ mol L^{-1})	Recovery (%)
(1)	4.8 ± 0.2	5.0	9.6 ± 0.2	96
(2)	5.2 ± 0.1	5.0	10.4 ± 0.2	104
(3)	5.0 ± 0.3	5.0	9.9 ± 0.3	98

Table 5
Determination of H_2O_2 in plant leaves (n = 5)

Sample	Sample content (μmol g^{-1})	Added in addition (μmol g^{-1})	Found total (μmol g^{-1})	Recovery (%)
Unpigmented				
(1)	0.43 ± 0.01	0.50	0.91 ± 0.03	96
(2)	0.60 ± 0.01	0.50	1.12 ± 0.01	104
(3)	0.52 ± 0.02	0.50	1.04 ± 0.01	104
Pigmented				
(1)	0.44 ± 0.02	0.50	0.93 ± 0.02	98
(2)	0.62 ± 0.01	0.50	1.11 ± 0.03	98
(3)	0.56 ± 0.01	0.50	1.04 ± 0.01	96

3.8. Sample analysis

Three different rainwater samples were determined and the standard additions method was used in the sample analysis procedure. The results were given in Table 3.

The serum samples were determined and the standard additional method was used in the sample analysis procedure. The results were shown in Table 4.

Cole leaves extracts were determined and comparison was made between 7 the unpigmented and pigmented samples (Table 5). Results indicated that there was no difference between the two types of samples, indicating that the plant pigments did not influence the determination in the near-infrared region. For these samples, only a previous extraction procedure was required.

4. Conclusion

A tricarbochlorocyanine dye (Cy.7.Cl) was successfully used as a new kind of fluorescence agent for near-infrared spectrofluorimetric determination of H_2O_2. The reaction is accelerated by HRP, allowing the effective automation of the method (FIA). The proposed method can effectively avoid the biological background interference. In addition, the method is simple, rapid, precise, sensitive and it is easily automated. The interference study and the recovery tests indicate that the method is suitable for the analysis of biological samples.

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