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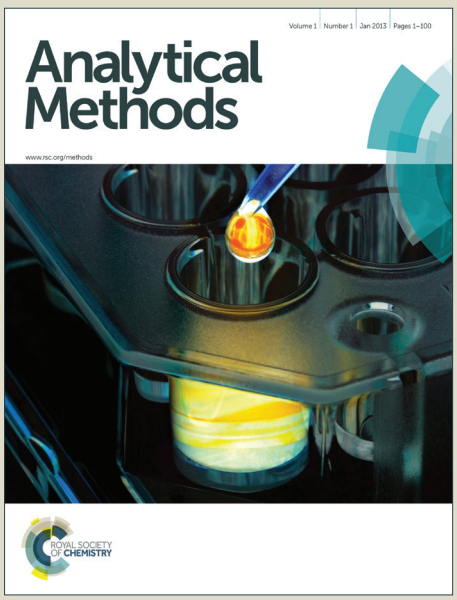

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A sensitive and rapid biosensor for determination of rhamnose based on catalytic effect on H_2O_2 oxidizing calcein

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Abstract In this paper, a new catalytic biosensor for rhamnose (Rha) detection has been developed based on combining the high sensitivity of fluorescence method and high selectivity of catalytic reaction. This rhamnose biosensor has the advantages over high sensitivity (the limit of detection (LOD) of 1.9×10^{-17} g mL^{-1}) and selectivity, non-radioactivity, non-incursion, safety, and it has been applied to determine trace Rha in cigarettes and jujubes, with the results agreed well with those obtained by high performance liquid chromatography (HPLC). At the same time, the component of calcein (R)-Rha also was analyzed by HPLC, mass spectrometry (MS) and nuclear magnetic resonance (NMR), and mechanism of catalytic biosensor for the determination of trace Rha was discussed.

Keywords Rhamnose; Calcein; Catalytic biosensor; Calcein-rhamnose condensate; Spectrum analysis

1. Introduction

Up to now, a new analysis method and instruments of research for sugars, DNA, protein and other biological macromolecules are facing new challenge [1-4]. As a kind of sugar, rhamnose (Rha) exists in plant, bacterial polysaccharides and plant glycosides [5]. Meanwhile, Rha has different functions including enhancing immunity, increasing energy, resisting fatigue, protecting liver and inhibiting osteoclasts, showing an intimate connection between Rha and human health [6-7]. Obviously, the rapid and sensitive determination of Rha has an important value in the field of physiological function research and clinical disease diagnosis based on the closely correlation of Rha content with human diseases.

In recent years, many methods have been reported for the determination of Rha, such as gas chromatography (GC) [8], high performance thin layer chromatography (HPLC) [9], reverse phase high-performance liquid chromatography [10], HPLC with indirect ultraviolet detection [11], high performance anion exchange chromatography with amperometric detection [12], capillary electrophoresis [13], automated enzymatic fluorometric assay [14] and so on. However, these methods still have some limitations. GC shows lower sensitivity. HPLC requires constant temperature and flow velocity, and is of low sensitivity [9]. Capillary electrophoresis requires the derivative treatments of test components [13]. Therefore, to develop a simple, rapid, cost-effective and sensitive method for the determination of Rha not only has broad application prospect, but also effectively promote the research development of the sugars physiological functions.. Recently, a novel colorimetric or fluorescent sensor for Cu^{2+} was developed based on combining the high sensitivity of catalytic reaction and the rapid response of sensor, showed notable advantage of the amplification effect for catalytic reaction [15-16].

We found that calcein (R) could not only emit strong and stable fluorescence, but also could be oxidized by H_2O_2 to form a non-fluorescence compound (R'), resulting in the quenching of fluorescence signal of R in this paper. Moreover, the ortho-hydrogen of phenolic hydroxyl in R took condensation reaction with Rha to produce non-fluorescence compound (R-Rha) causing the fluorescence signal of R to further quench, and R-Rha was oxidized by H_2O_2 to form R' and Rha, bringing about the sharp fluorescence signal quenching of R. Based on the facts above, a new catalytic biosensor for Rha detection based on its strong catalytic effect on H_2O_2 oxidizing R has been designed, with a LOD of 1.9×10^{-17} g mL^{-1}, which is 6.6×10^8 times lower than the lowest of HPLC (2.0×10^{-8}g g^{-1}) [9]. reported before. This catalytic biosensor has been used to detect the Rha in actual samples with satisfactory results due to it has some advantages, such as selectivity, selectivity, simplicity, rapid and low-cost. What's more, catalytic biosensor for Rha detection has rarely been reported yet. This study not only provides a new way for improves the sensitivity of fluorescence method, but also promotes the applications of biosensor**,** catalytic kinetic analysis and development of trace analysis.

2. Experimental

2.1 Apparatus and reagents

Fluorescence measurements were carried out on an LS-55 fluorescence spectrophotometer (Perkin-Elmer (Norwalk, CT 06859-0243, USA). The instrument's main parameters are as follows: band pass *E*x = *E*m = 10.0 nm and scan speed 1500 nm min^{-1}. AE240 electronic analytical balance (Mettler-Toledo Instruments Company Limited), pHS-3B precision acidometer (Shanghai Medical Laser Instrument Plant), high-press constant-current pump (P200, produced by Dalianyilite

Company, in China), the refractive index detector (Waters 450) and the chromatographic column (HYDERSIR-NH_2, produced by Dalianyilite Company) were also used in the experiment.

Preparation of Rha working solution: 0.08000 g and 0.01000 g Rha were dissolved in water and diluted to 10.0 mL, respectively. The concentration of stock solution was 8.0 and 1.0 mg mL^{-1}. It was gradually diluted to 0.80 pg mL^{-1}, 100.00, 10.00 and 1.00 fg mL^{-1} with water as work solution, respectively. 1.0×10^{-4} mol L^{-1} R and 3.0% (V/V) H_2O_2 solution were also used in this experiment. All the reagents were analytical reagents except that Rha was primary standard reagent. The water was prepared by thrice sub-boiling distillation.

2.2 Experimental method

Proper amount of Rha, 0.50 mL R and 1.00 mL H_2O_2 were added into a 25- mL colorimetric tube, diluted to 25 mL with water, and mixed homogeneously. The mixture was kept at 70 °C for 10 min in a water bath, cooled by flowing water for 5 min. At the same time, a reagent blank experiment (without Rha) was also conducted. The fluorescence intensity of test solution (F) and reagent blank (F_0) was directly measured at 488.3/513.4 nm ($\lambda_{ex}^{max}/\lambda_{em}^{max}$). Then, ΔF ($=F_0-F$) was calculated.

2.3 HPLC, MS and NMR of R-Rha constitute

1.00 mL of 0.80 pg mL^{-1} Rha and 50.00 mL R were kept at 70 °C for 10 min in water bath, and they were cooled to obtain R-Rha crystal. 0.10 mg R-Rha was dissolved in water and separated with high performance sugar analytical column (WAT 044355, 250 mm × 4.6 mm i.d., 4 μm) and acetonitrile-ethyl acetate-water (60:25:15) was used as mobile phase. Then, degassed by an ultrasonic apparatus and filtrated by filter-film (0.45 μm). R-Rha was separated by HPLC (The flow velocity was 0.6 mL min^{-1}, and the injection volume was 10.0 μL.), and detected by fast atom bombardment mass spectroscopy (FABMS), ^{1}HNMR and ^{13}CNMR.

3. Results and discussion

3.1Mechanism for the determination of Rha

In order to demonstrate the feasibility of the sensing mechanism for the detection of Rha, the fluorescence spectra of R-H_2O_2-Rha system were scanned. As shown in Fig. 1, R could emit strong and stable fluorescence signal ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 493.8 /514.4 nm, F =355.3, Fig. 1, curve 1.1') after incubating at 70 ℃ for 10 min and its fluorescence signal could be quenched by Rha ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 488.3/514.4 nm, F =189.1, curve 3.3').

Fig. 1 Fluorescence spectra of R-H_2O_2-Rha system (Curves 1, 2, 3 and 4 were the excitation spectra , curves 1', 2', 3' and 4' were the emission spectra.)

1.1' 0.50 mL R 2.2' 1.1'+1.00 mL H_2O_2 3.3' 1.1'+ 300.0 fg Rha 4.4' 2.2'+ 300.0 fg Rha

In the presence of H_2O_2, the fluorescence signal of R quenched ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 488.3/513.7 nm, F =192.3, Fig. 1, curve 2.2') since H_2O_2 could oxidize R to form non-fluorescence compound R'. The reaction can be expressed as follows (Scheme 1).

Scheme 1 Reaction of H_2O_2 oxidizing R

When Rha was added, the fluorescence signal of R sharply quenched by Rha ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 488.3/513.4 nm, F = 95.9, ΔF = 96.4, Fig. 1, curve 4.4') due to the formation of non-fluorescence conensate R-Rha in condensation reaction between the ortho-hydrogen of phenolic hydroxyl in R

and Rha (Scheme 2).

Scheme 2 Condensation reaction between R and Rha

In order to prove the possibility of Rha to react with R and form R-Rha, the structure of R-Rha was analyzed with HPLC, FABMS and NMR. Results in the figure of FABMS indicate the existence of R (m/z 620), Rha (m/z 180) and R-Rha (m/z 800), and the appearance time of Rha sample (6.186 min) was the same as that of standard Rha as shown in the figure of HPLC. All these facts proved the existence of Rha and R in the condensate R-Rha. Otherwise, the conclusion that Rha existed in the product could also be drawn from the spectra data of ^{1}H NMR [(D_2O, δ1.27 (CH_3-), δ3.2-4.1 (2'H, 3'H, 4'H, 5'H), δ4.81 (β, 1'H), δ5.1 (α, 1'H)) and ^{13}C NMR [(D_2O) δ17.6 (CN_3-), δ69 (α, 5'C), δ72.7 (β, 5'C), δ70.8 (α, 3'C), δ71.6 (α, 2'C), δ72.2 (β, 2'C), δ73 (α, 4'C), δ73.5 (β, 1'C), δ94.3 (β, 1'C), δ94.8 (α, 1'C), δ68.7 (β, 4'C)], which also proved that the possibility of Rha reacting with R to form R-Rha

The fluorescence signal of R sharply quenched resulting from H_2O_2 oxidizing R-Rha to be R' and Rha, indicating the remarkable catalytic effect of Rha on H_2O_2 oxidizing R. And the possible reaction is expressed in Scheme 3.

Scheme 3 Reaction between R-Rha and H_2O_2

0.50 mL H_2O_2 was added into 0.10 mg R-Rha, heated at 70 ℃ for 10 min, and then cooled. The product was separated by HPLC and analyzed by mass spectrometer. Results show that there were two chromatographic peaks at 6.209 min for Rha and 21.307 min for R'. Besides, the molecular ion

peaks of 180 $[M]^+$ and 620 $[M]^+$ in the figure of FABMS further proved the existence of R'and Rha. The facts indicated the possibility that H_2O_2 oxidized R-Rha to form R' and Rha.

The ΔF of the system was linear to the content of Rha. Thus, trace Rha could be determined by catalytic biosensor..

3.2 Optimum measurement conditions

For the system containing 4.0 fg Rha mL^{-1}, the effects of the dosage and concentrations of reagents, luminescence substances, oxidants, reaction time and temperature, reaction acidity, and standing time on the ΔF of the system were examined in a by univariate approach (Table 1**S**, **Fig. 1S-6S**), respectively.

Though the ΔF of the system were high when FITC and Rhod.6G were chosen as luminescence substances they were still lower than that of R. Further investigations found that with the increasing of the concentration and dosage of R, the ΔF of the system enhanced gradually. When the concentration or dosage of R was 0.50 mL of 1.0×10^{-4} mol L^{-1}, the ΔF of the system reached the maximum due to the formation of non-fluorescence conensate R-Rha in condensation reaction between the ortho-hydrogen of phenolic hydroxyl in R and Rha.

Compared with KIO_4, $K_2S_2O_8$, $KClO_3$ and $KBrO_3$, H_2O_2 had the largest effect on the ΔF of the system. The main reason might be the strongest oxidity of H_2O_2. With the increasing of the concentration and dosage of H_2O_2, the ΔF of the system enhanced gradually. When the concentration or dosage of H_2O_2 was 1.00 mL of 3.0 %, the ΔF of the system reached the maximum.

As the reaction time and temperature increased, the ΔF of the system gradually enhanced, might resulting from increasing of the catalytic ability of Rha gradually. When the reaction temperature and

time were 70 °C and 10 min, respectively, the Δ*F* of the system reached the maximum, whose reason might that the catalytic ability of Rha reached the peak. Henceforth, with the reaction temperature and time continuously increasing, the Δ*F* of the system decreased due to the decreasing of catalytic ability of Rha gradually.

The Δ*F* of the system linearly enhanced with the increasing of pH in the range of 5.00-6.65, while it reached the maximum and remained stable when the pH was in the range of 6.65−7.50. The reason might be that the catalytic reaction rate of Rha reached the maximum.

The Δ*F* of the system almost stayed invariable and had good repeatability within 15-45 min. But the Δ*F* of the system declined when the standing time was over 45 min, possibly due to the decomposition of R-Rha.

Therefore, the Δ*F* of the system reached the maximum and remained stable when 0.50 mL of 1.00×10^{-4} mol L^{-1} R and 1.00 mL of 3.0% H_2O_2 were used, the reaction temperature was 70 °C and the time was 10 min, pH was 7.10. Under the optimal conditions above, the Δ*F* of the system almost remained unchanged within 15-45 min after being cooled by flowing water for 5 min, indicating the method had good repeatability.

3.3 Working curve, linear range and LOD

Under the optimum conditions, the Δ*F* of the system had linear relationship with the content of Rha (**Fig. 2**) .The linear range, regression equation of the working curve, correlation coefficient (*r*), relative standard deviation (RSD, 0.050 and 12.0 fg mL^{-1} Rha were determined in parallel for 6 times), LOD (calculated by 3Sb/*k* which referred to the quotient between thrice of the standard deviation of blank reagent and the slope of the working curve, *n* = 11) and LOQ (calculated by 10Sb/*k*) of the method were compared with those of references [8, 9, 11]. Results are listed in **Table 1**.

Table 1 Compare with some methods for determination of Rha

Table 1 showed that the LOD was 1.9×10^{-17} g mL^{-1} and linear range was 5.0×10^{-17}-$1.2.\times10^{-14}$ g mL^{-1}, showing the higher sensitivity and wider linear range of the method than those in Ref. [9]. There are two reasons for higher sensitivity of this sensor. Firstly, the formation of non-fluorescence conensate R-Rha in condensation reaction between the ortho-hydrogen of phenolic hydroxyl in R and Rha, showing that R-Rha had activating effect on the reaction of H_2O_2 oxidizing R; Secondly, the catalytic effect of Rha on the reaction of H_2O_2 oxidizing R greatly improves ΔF value, showing the signal amplification of catalytic reaction.

Fig. 2 Fluorescence change (ΔF) titrated with Rha under the optimum conditions

3.4 Selectivity of the catalytic biosensor

To evaluate the selectivity of catalytic biosensor towards the sample containing 4.00 fg Rha mL^{-1}, a series of coexistent materials in tobacco and jujube were investigated by this method under the optimum measurement conditions. When the relative error (Er) exceeded ± 5%, each materials is considered as interfering agent. Results show that the allowable concentrations (pg mL^{-1}) of most materials are high: 450 for aspartate, 430 for glutamic acid, 420 for lysine and tryptophan, 410 for ascorbic acid, 30 for mannose and xylose, 25 for glucose and fructose as well as 24 for galactose, showing better selectivity of this catalytic biosensor. The reason might be that –CHO and $–CH_2OH$ in glucose, mannose, xylose, fructose and galactose were oxidized by H_2O_2 to be tetrahydroxy diacid, which hindered the condensation reaction with ortho-hydrogen of phenolic hydroxyl in R.

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3.5 Analytic application

According to the method in Ref. [9], 1.0 g (± 0.01 mg) of tobacco sample with particle diameter ranging from 0.30 nm to 0.45 mm was weighed. Then, the samples were transferred into the beaker and 25.00 mL water was added, shaking with ultrasonic oscillator at 40 ℃ for 40 min. After being filtrated and washed, the filtrate was gained and diluted to 100.00 mL. 5.00 mL test solution was taken, and passed through pre-activated reversed phase C-18 solid extraction column at the speed of 1.0 mL min^{-1}. The initial 2.00 mL effluent solution was discarded and the rest 3.00 mL solution was collected. Then, the solution was filtrated with 0.45 μm water system filter membrane and 1.00 mL filtrate was diluted to 250.00 mL. And then 1.00 mL of the test solution was diluted to 1000.00 mL for use. The reversed phase C-18 solid extraction column activated by methanol and water could remove aldehyde, ketone, lactone and organic acid in the sample solution [17], which effectively protected the C-18 solid extraction column and prolonged its service life.

According to the method in Ref. [18], proper amount of jujube was pitted and dried in a vacuum at 50 ℃, and then placed in a desiccator until being cooled. The desiccated jujube was grinded and mixed homogeneously, and jujube powder was obtained. 1.0 g (± 0.01 mg) jujube powder with particle diameter ranging from 0.30 nm to 0.45 mm and 100 mL of 95% ethanol were added into the beaker (the total monosaccharide in the jujube could be extracted with 95% ethanol [18]). Then, the mixture was extracted by ultrasonic oscillator at 50 ℃ for 120 min, and then was separated by centrifuge. At last, the gained solution was filtrated. The steps were repeated for three times. The filtrate was collected, decompressed and diluted to 250.00 mL with water. 5.00 mL solution was treated according to the treatment of cigarettes. And 1.00 mL of the test solution was diluted to 250.00 mL, and then 1.00 mL solution was taken and diluted gradually to 2000.00 mL for use. 1.00

mL of test solution was taken out for Rha detection in cigarettes and jujubes according to the experimental method and HPLC, respectively. Meanwhile, a standard addition recovery experiment of Rha was conducted and Er was calculated. The results are listed in Table 2.

Table 2 Analytical results of Rha in samples

This catalytic biosensor with high sensitivity, good repeatability and high selectivity has been applied to determine the content of Rha in cigarettes and jujubes, the results were in good agreement with those obtained by HPLC, indicating has significance and academic research value in life sciences.

4. Conclusion

In this work, a catalytic biosensor with signal amplification for Rha detection has been proposed based on the fact that Rha could accelerate the reaction between R and H_2O_2, caused rapid response of the ΔF to [Rha]. This rapid, simple, sensitive and selective biosensor not only displays potential application prospect in biological analysis, but also indicates notable advantage of combination between high sensitivity of biosensor and signal amplification of catalytic reaction and effectively promoted the development and applications of the R, biosensor and catalytic kinetic analysis.

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Table captions

Table 1 Compare with some methods for determination of Rha

Table 2 Analytical results of Rha in samples

Table 1 Compare with some methods for determination of Rha

Method	Linear range (g mL^{-1})	Regression equation of the working curve	r	RSD (%)	LOD (g mL^{-1})	LOQ (g mL^{-1})
Biosensor	5.0×10^{-17}-$1.2.\times10^{-14}$	ΔF = 2.346 +7.810 C_{Rha} (fg mL^{-1}), S_b = 0.050	0.9996	2.1-3.2	1.9×10^{-17}	6.3×10^{-17}
GC[8]	6.1×10^{-6}-$2.4.\times10^{-5}$	Y = 3.125×10^{4}X-2.0×10^{6}(mg mL^{-1})	0.9993		6.1×10^{-6}	
HPLC[9]	2.0×10^{-8}-3.2×10^{-7}	Y = 5. 569X (ng g^{-1})-1. 6	0.9998	1.0-2.0	2.0×10^{-8} (g g^{-1})	
HPLC with indirect ultraviolet detection [11]	9.0×10^{-7}-2.7 $\times10^{-4}$	Y = 0.0526X (μM)- 0.0116	0.9998	1.8	2.9×10^{-7}	

Table 2 Analytical results of Rha in samples

Cigarettes and jujubes	Found ($\mu g\ g^{-1}$)	RSD (%, $n = 6$)	Added ($\mu g\ g^{-1}$)	Found ($\mu g\ g^{-1}$)	Obtained ($\mu g\ g^{-1}$)	Recovery (%)	HPLC ($\mu g\ g^{-1}$)	Er (%)
Hong Mei cigarette	0.356	4.1	0.050	0.405	0.0490	98.6	0.368	−3.3
Zhong Hua cigarette	0.368	3.5	0.050	0.417	0.0489	97.8	0.375	−1.9
Hong Ta Shan cigarette	0.352	2.8	0.050	0.403	0.0510	102.0	0.343	+2.6
Gong He Xin Xi cigarette	0.361	1.9	0.050	0.411	0.0500	100.0	0.352	+2.6
Qi Pi Lang cigarette	0.355	3.2	0.050	0.405	0.0496	99.2	0.362	−1.9
Nan Jing cigarette	0.352	2.6	0.050	0.400	0.0484	96.8	0.364	−3.3
Da Qian Men cigarette	0.378	3.0	0.050	0.429	0.0506	102.0	0.361	+4.7
Yu Xi cigarette	0.402	2.1	0.050	0.451	0.0490	98.0	0.412	−2.4
Chen Xiang cigarette	0.351	1.8	0.050	0.399	0.0480	96.0	0.366	−4.1
Mu Dan cigarette	0.347	3.7	0.050	0.396	0.0493	98.0	0.358	−3.1
Zhong Nan Hai cigarette	0.369	4.2	0.050	0.420	0.051	102.0	0.357	+3.4
Hebei jujube	1.33	1.5	0.14	1.466	0.136	97.1	1.40	−5.0
Shandong Jujube	1.09	2.0	0.11	1.201	0.111	101.0	1.05	+3.8
Shanxi jujube	1.21	1.8	0.12	1.329	0.119	99.2	1.26	−4.0

Figrue captions

Fig. 1 Fluorescence spectra of R-H_2O_2-Rha system (Curves 1, 2, 3 and 4 were the excitation spectra , curves 1', 2', 3' and 4' were the emission spectra.)

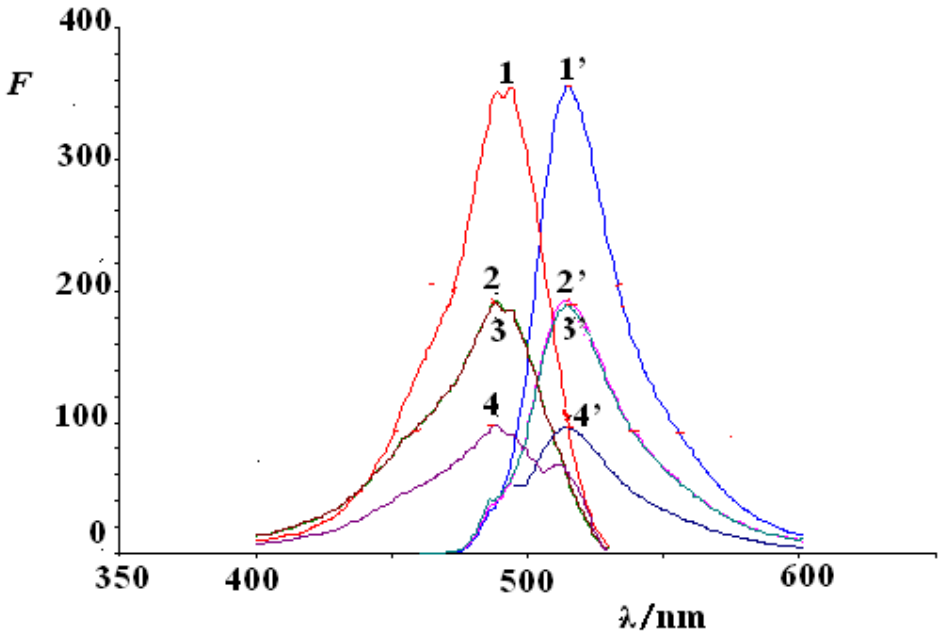
1.1' 0.50 mL R 2.2' 1.1'+1.00 mL H_2O_2 3.3' 1.1'+ 300.0 fg Rha 4.4' 2.2'+ 300.0 fg Rha

Scheme 1 Reaction of H_2O_2 oxidizing R

Scheme 2 Condensation reaction between R and Rha

Scheme 3 Reaction between R-Rha and H_2O_2

Fig. 2 Fluorescence change (ΔF) titrated with Rha under the optimum conditions



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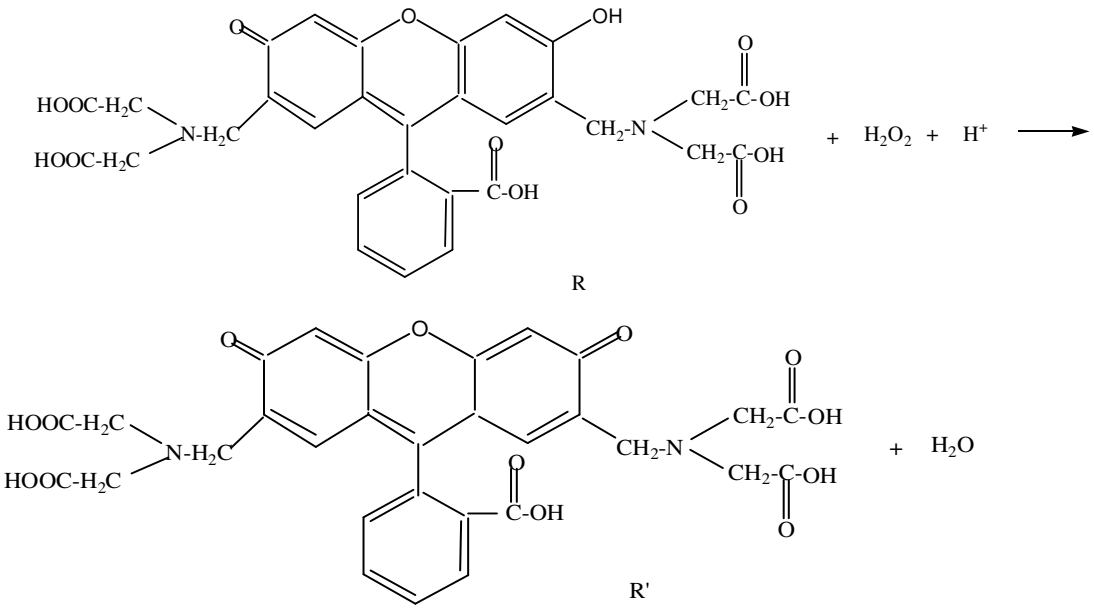
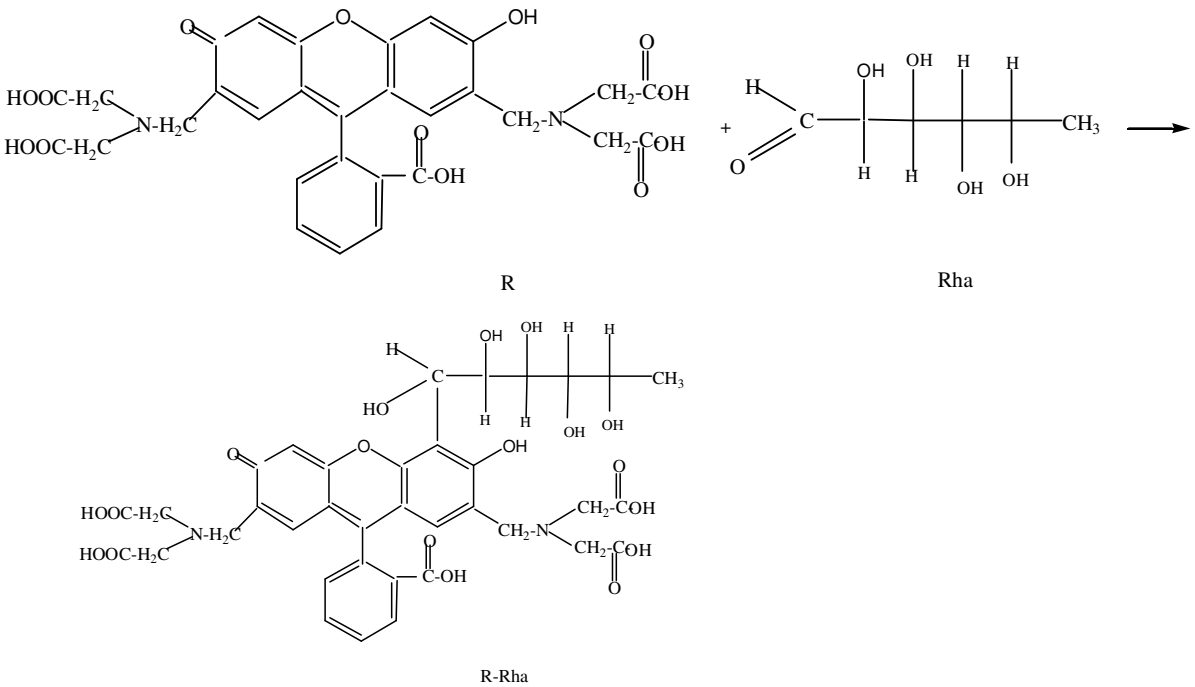
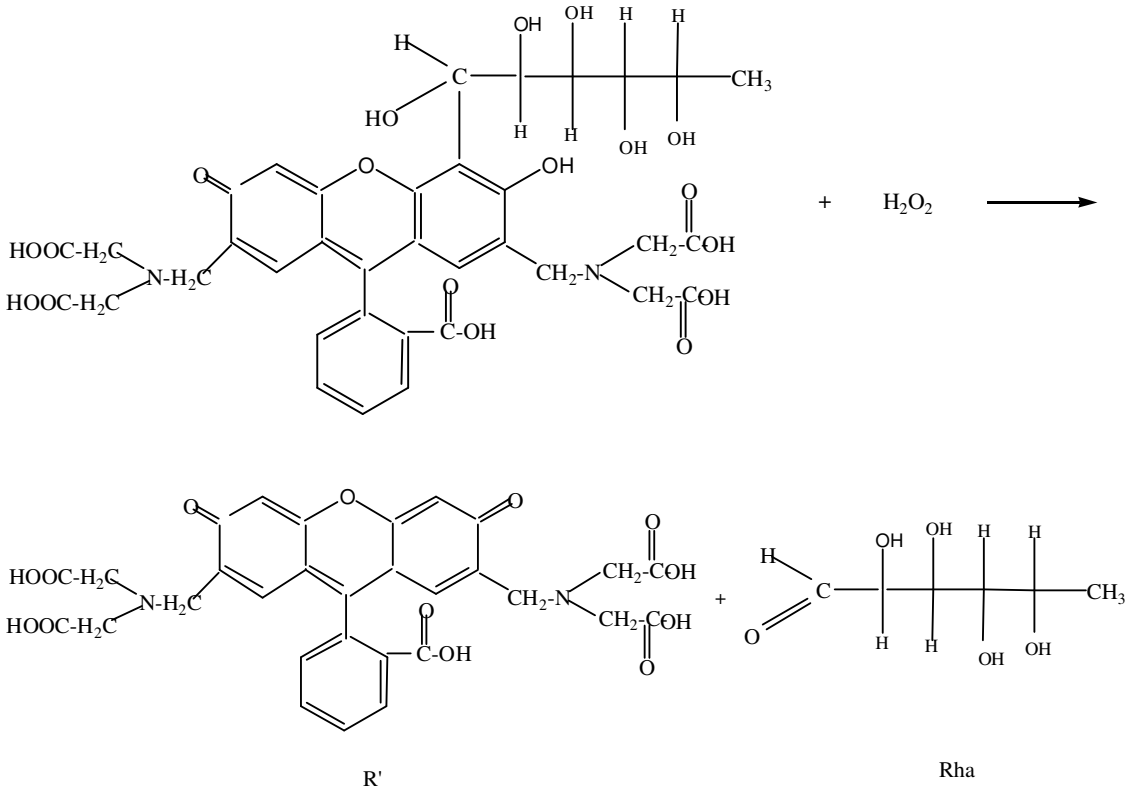


Scheme 1 Reaction of H_2O_2 oxidizing R



Scheme 2 Condensation reaction between R and Rha



Scheme 3 Reaction between R-Rha and H_2O_2

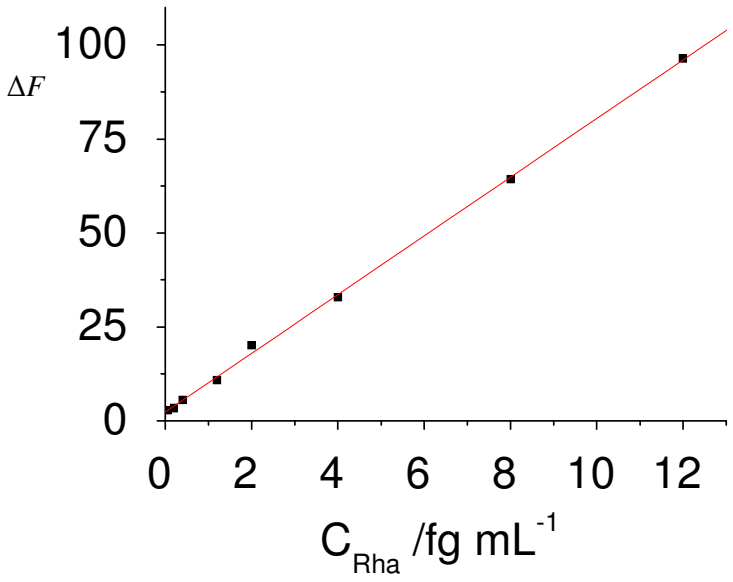


Fig. 2 Fluorescence change (ΔF) titrated with Rha under the optimum conditions