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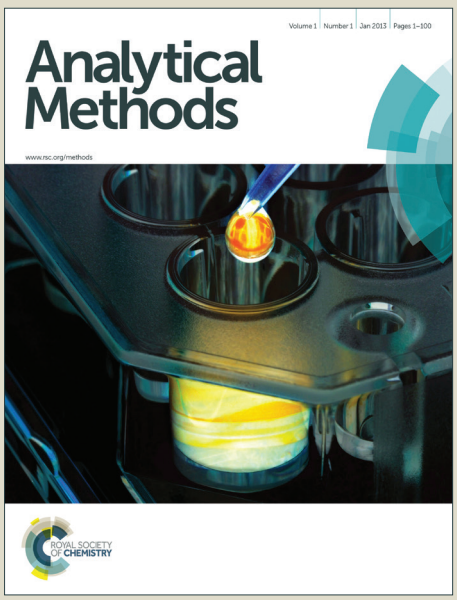

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Ultra-sensitive solid substrate-room temperature phosphorimetry for colchicine detection based on its catalytic effect on H_2O_2 oxidize acridine yellow

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Abstract A new solid substrate-room temperature phosphorimetry (SS-RTP) for colchicine (COL) detection has been established based on its strong catalytic effect on H_2O_2 oxidize acridine yellow (AY), which caused the room temperature phosphorimetry (RTP) of AY to quench sharply. This high sensitive (The limit of quantification (LOQ) was $3.1\times10^{-1\ 3}$ g mL^{-1}), accurate and selective SS-RTP has been successfully applied to the COL detection in the human serum and tea samples with the results agreeing well with high performance liquid chromatography (HPLC) , the results were coincident with those of ultra fast liquid chromatography-tandem mass spectrometry (UFLC-MS/MS) method.. The activation energy and the reaction rate constant of catalytic reaction were 40.53 kJ mol^{-1} and 3.97×10^{-4} s^{-1}, respectively. In addition, the reaction mechanism of catalytic SS-RTP for COL detection was also discussed using infrared spectrum (IR), nuclear magnetic resonance (NMR) and electron impact mass spectra (EIMS).

Keywords: Colchicine, Acridine yellow, Hydrogen peroxide, Catalytic solid substrate-room temperature phosphorimetry

1. Introduction

Colchicine (COL) not only can be used to treat gouty and some malignant tumors [1], but also can be used to predict and treat familial mediterranean fever, kidney disease, some dermatosis and fibrotic disease and so on, which shows better application foreground [2]. Medical reports indicate that for adults, more than 0.8 mg/kg colchicine is routinely fatal, suggesting a lethal dose of 40–60 mg for a 50–75 kg adult [3-4]. As known colchicine's genotoxicity with regards to in vitro and in vivo systems even at low concentrations. Obviously, COL is closely related to human diseases, and it has important application value in clinic.

In recent years, there are many methods for COL detection, such as fluorimetry [5], HPLC/MS [6], HPLC [7], UFLC-MS/MS [8], electrochemical method [9], TLC-densitometry [10] and so on. However, HPLC/MS and HPLC needs organic solvent to extract and the operation is complicated. Electrochemistry method, TLC-densitometry and fluorimetry are difficult to meet the needs of determination of COL in the research of pharmacokinetics due to their low sensitivity [8]. Although the sensitivity of UFLC-MS/MS is high, the instruments used are expensive and the analysis cost is high, which limits its application range. Thus, to find a high sensitive, simple, fast, and low analysis cost method for COL detection has significant meaning and academic value.

Compared with fluorimetry, phosphorimetry has many merits such as larger Stoke's shift, easier to reduce the interference of background fluorescence and scattering light, longer lifetime and better selectivity etc. Taking advantage of these characteristics, lots of phosphorimetry have been developed for the detection of toxin ions [11-12], biological active substances [13-18], organic molecules [19-21], showing the potential application prospects of phosphorimetry.

In the study, we found that AY could emit strong and stable signal RTP on polyamide membrane (PAM) solid substrate with Pb^{2+} as ion perturber and COL could catalyze the reaction of H_2O_2 oxidizing AY at 100 °C for 10 min, which resulting in that the RTP signal of AY quenching and the value of ΔI_p has good linear relationship with COL. Thus, catalytic SS-RTP for COL detection has been developed. The limit of detection (LOD was 9.3×10^{-14} g mL^{-1}) of this method was lower than that (5.0×10^{-11} g mL^{-1}) of UFLC-MS/MS [8]. This sensitive, accurate, selective and repeatable SS-RTP was suitable for COL detection in human serum, and to our knowledge, catalytic SS-RTP for COL detection based on the catalyzing H_2O_2 oxidizing AY has not been reported yet. The establishment and application of the new method and the research of reaction mechanism greatly

promoted the research development of the detection technique of COL.

2. Experimental

2.1 Apparatus and Reagents

Phosphorescent measurements were carried out on a LS-55 luminescence spectrophotometer with a solid surface analysis apparatus (Perkin Element Corporation, U.S.A.). The instrument's main parameters are as follows: delay time 0.1ms; gate time 2.0 ms; cycle time 20 ms; flash count 1; excitation slit 10 nm; emission slit 15 nm; scan speed 1500 nm min^{-1}. KQ-250B ultrasonic washing machine (Kunshan Ultrasonic Machine Corporation, China), AE240 electronic analytical balance (Mettler-Toledo Instruments Corporation, China) and a 0.50-μL flat head micro-injector (Shanghai Medical Laser Instrument Plant, China) were used.

COL (National institute for the control of pharmaceutical and biological products) working solutions: 10.0 μg $mL^{-1}$of COL stock solutions were gradually diluted to 1000.0,100.0 and 10.0 pg mL^{-1}, respectively. 1.0×10^{-4} mol L^{-1} AY, 2 % (w/v) H_2O_2 solution and 1.00 mol L^{-1} $Pb(Ac)_2$ were also prepared. All reagents were analytical regent grade except that COL was standard reagent. The water was prepared by thrice sub-boiling distillation.

Filter paper used was purchased from Xinhua Paper Corporation (Hangzhou, China). PAM, acetyl cellulose membrane (ACM) and nitric cellulose membrane (NCM) were purchased from Luqiaosijia Biochemical Plastic Plant. The paper sheer were pre-cut into wafers (Diameter is 4.0 mm.) for use.

2.2 Experimental method

The proper amount of COL working solution, 1.00 mL AY and 4.00 mL H_2O_2 were placed in a 25.0 mL colorimetric tube, and diluted to 25.0 mL with water and mixed homogeneously. The mixture was kept at 100 °C for 10 min, cooled cooled by flowing water for 5 min. PAM was immersed into 1.0 mol L^{-1} Pb^{2+} solution for 10 s, and then dried at 90 ± 1 °C for 2 min. 0.40 μL test solution and blank solution was suspended at the center of each sheet (Diameter is 4.0 mm.) by a 0.50 μL flat head micro-injector, respectively, then the wafer was dried at 90 ± 1 °C for 2 min again. The phosphorescence intensity of the reagent blank (I_{p0}) and the phosphorescence intensity of catalyze reaction solution (I_p) at 442 nm and 507 nm were determined, respectively Then ΔI_p (= I_{p0} – I_p) was calculated.

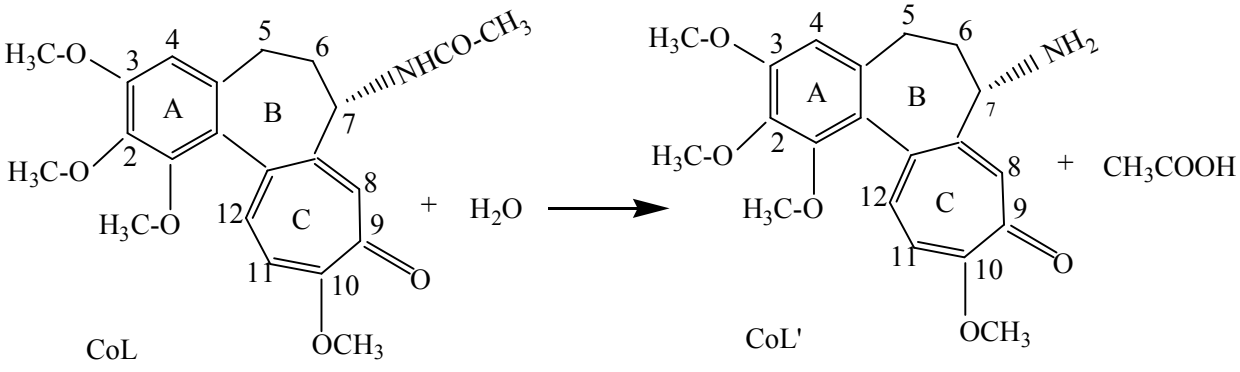
2.3 HPLC, IR, EIMS and NMR of CoL'-AY constitute

100.00 mL of 1.00 ng mL^{-1} COL and 100.00 mL AY were kept at 100 °C for 10 min in water bath, and they were cooled to obtain COL'-H…N-AY crystal. 0.010 mg COL'-H…N-AY was dissolved in water and separated with high performance analytical column (Zorbax Extend-C18, 150 mm × 4.6 mm., 5 μm) and CH_3OH: 10 $mmolL^{-1}$ $CH_3COONH_4^{+\cdot}$(60:40) was used as mobile phase. Then, degassed by an ultrasonic apparatus and filtrated by filter-film (0.45 μm). COL'-H…N-AY was separated by HPLC (The flow velocity was 1.10 mL min^{-1}, column temperature was 40 °C and the injection volume was 20.0 μl.), and COL'-H…N-AY was obtained. 10.00 mL of 100.0 pg mL^{-1} COL was added to a 25–mL colorimetric tube, diluted to 25.0 mL with water, and kept at 100 °C for 10 min in water bath, the mixture was by flowing water for 5 min. The mixture was separated by HPLC, and COL' was obtained. IR (The sample preparation by pressed disc method with KBr.), EIMS and ^1H NMR were scanned, respectively..

3. Results and discussion

3.1 Reaction mechanism for the determination of COL

AY could emit strong and stable RTP signal using Pb^{2+} as ion perturber. When1000.0 pg COL was added into AY-H_2O_2 system, COL was hydrolyzed into COL' under the conditions of 100 °C and 10 min [22] (**Scheme 1**).

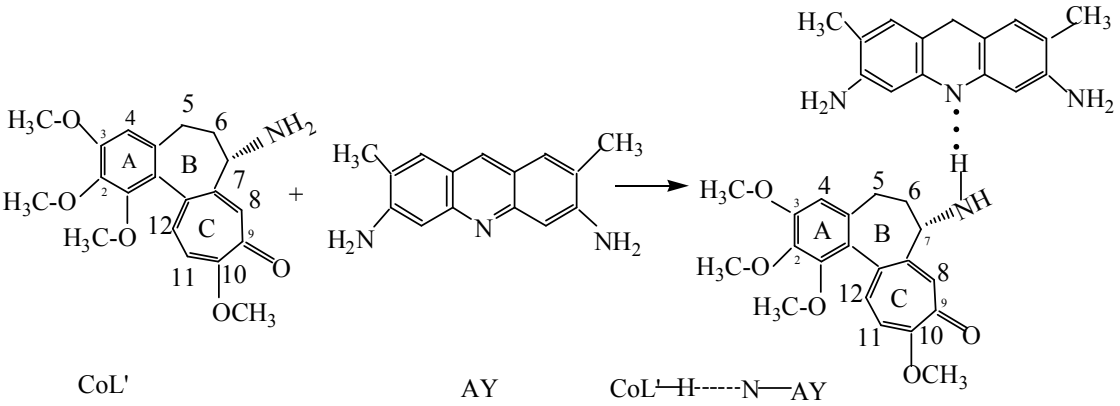


Scheme 1 Hydrolyzed reaction of COL

In order to prove the probability of hydrolyzed reaction of CO L occurred, the IR of COL and

COL'were scanned by Nicolet-360 infrared spectrometer (KBr pellet) ranging from 200 cm^{-1} to 4000 cm^{-1}. The spectra data of IR for of COL in **Table 1S, which** showed that $–OCH_3$, –N–H, $–CH_3$ peaks located at 2850,3300 and 3180 cm^{-1}, and two $\nu_{C=O}$ peak at 1665 and 1640 cm^{-1}, while the characteristic absorption peak of $\nu_{C=O}$ peak at 1665 cm^{-1} in COL' disappeared and there was one $-NH_2$ peak located at 3450 cm^{-1}, which proved that the the probability of hydrolyzed reaction of COL occurred to form COL'.

Being similar to the effect of DNA [23], nitrogen atom with strong ability to coordinate in AY could react with the hydrogen of bases in COL' molecule to form the hydrogen bond and then turn into non-phosphorescence compounds (COL'-H…N-AY, **Scheme 2**).The results led the RTP signal of AY to quench.

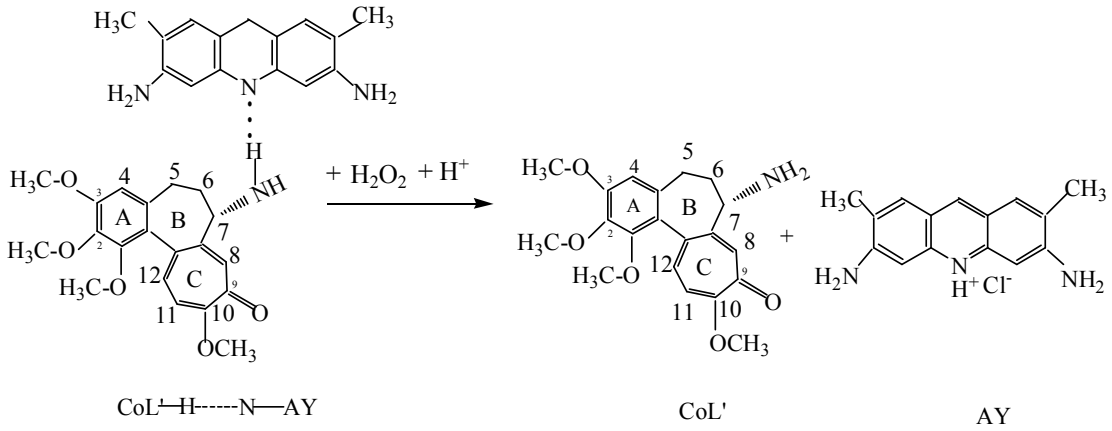


Scheme 2 Reaction between COL' and AY

In order to prove the possibility of COL' to react with AY and form COL'-H…N-AY, the structure of COL'-H…N-AY was analyzed with HPLC, EIMS and ^1H NMR. The spectra data of HPLC indicate the appearance time of CoL' and AY samples (2.80 min, 2.55 min) was the same as that of standard CoL'. There was characteristic absorption peaks of CoL' and N-AY complex in the IR spectrum of COL'-H…N-AY. All these facts proved the existence of CoL' and N-AY in the condensate COL'-H…N-AY. At the same time, When *m*/*Z* were 594 $[M+1]^+$, there were mass peaks appeared at 237 (100) and 357 (12.6) on the EIMS. The peak at *m*/*Z* 357, *m*/*Z* 237 was for CoL', N-AY which agreed with their theory relative molecular mass (357.6, 237.9), concluding that coordination ratio of CoL' and N-AY was 1:1. The mass spectra peak of $[M+1]^+$ at 594 (7.4) was the product of CoL' and N-AY with the ratio of 1:1, this value corresponds closely to the calculated

molecular mass (594.5) of the desired product. Besides, the difference between 594 and 357 was 237, which was just the 1 times of the relative molecular mass of N-AY molecule, indicating that the ratio of CoL', N-AY was 1:1. In addition, there was characteristic absorption peaks of CoL' (7.49(s, 1H; H-8) , 7.41 (d, 1H, 10. 4Hz; H-12) , 6.86 (d, 1H, 10. 4Hz, H-11) , 6.53 (s, 1H, H-4) , 6.58 (s, 2H; NH_2) , 4.68 (m, 1H; H-7) , 3.91 (s, 6H, 2×OCH_3), 3.60 (s, 3H,OCH_3), 1.82, 2.41 (m, 4H, 2×CH_2)) and N-AY(10.13 (2H , s , -N- H), 8.89-7.62 (15H , m , Ar - H) ; 2.58 (6H , s , 3.5 - CH_3)) complex in the ^{1}H NMR spectrum of COL'-H…N-AY(7.45 (s, 1H; H-8) , 7.36 (d, 1H, 10. 4Hz; H-12) , 6.82 (d, 1H, 10. 4Hz, H-11) , 6.49 (s, 1H, H-4) , 6. 55 (s, 2H; NH_2) , 4.63 (m, 1H; H-7) , 3.87 (s, 6H, 2×OCH_3), 3.55 (s, 3H,OCH_3), 1.77, 2.36 (m, 4H, 2×CH_2), 10.09 (2H , s , - N - H), 8.85-6.55 (15H , m , Ar-H) ; 2.52 (6H , s , 3.2 -CH_3),), which also proved that the possibility of CoL' reacting with N-AY to form of COL'-H…N-AY. Above-mentioned the spectra data of HPLC, IR, ^{1}HNMR and EIMS proved that the possibility of COL' reacting with AY to form COL'-H…N-AY.

In the presence of H_2O_2, COL'-H…N-AY was oxidized by H_2O_2 to form COL'and AY (**Scheme 3**). The reaction can be expressed as was as follows:



Scheme 3 Reaction of H_2O_2 oxiding COL'-H…N-AY

COL' reacted with AY to form the non-phosphorescence compounds COL'-H…N-AY (**Scheme 2**), which led the RTP of system to quench sharply, the ΔI_p (116.6) was 10.7 times larger than that (10.9) of non-catalytic reaction. It showed that the catalytic reaction of COL had an amplification effect on measure signal. Thus, trace COL could be determined by catalytic SS-RTP.

In order to prove the probability of catalytic reachting mechanism of H_2O_2 oxidizing AY by COL, the apparent activation energy (E) and reaction rate constant (k) were investigated under the optimal condition above. For the system containing 4.8 fg spot^{-1} COL, examine the effects of different reaction time (t) and temperature (T) on signal/background ratio (I_{p0}/I_p) of phosphorescence. First, fixing the reaction time at 10 min, – log[log Ip/ Ip_0] is proportional to 1/T when the temperature is within 60–100°C, the regression equation can be expressed as – log[logI_{p0}/I_p] = 1.819×(1/ T)×1000 –3.903, r = 0.9966, when the temperature is 100°C the ΔI_p reached maximum with E of 40.53 kJ mol^{-1}; Second, fixing the reaction temperature at 100°C, ln(I_{p0}/I_p) is proportional to reaction time (t) when the time is within 4–10 min, indicating that it was a first order reaction, the regression equation can be expressed as ln(I_{p0}/I_p) = –0.0732 + 0.0306 t (min), r = 0.9975, when the reaction time is 10 min, ΔI_p reached maximum with k of 3.97×10^{-4} s^{-1}. So the optimum reaction temperature and time for SS-RTP measurement is 100 ^{o}C and 20 min, respectively. These facts above not only confirmed the catalysis of COL on oxidation reaction between the H_2O_2 and AY, but also demonstrated the probability of reaction mechanism for the determination of COL.

3.2 Phosphorescence spectra

The phosphorescence spectra of COL-H_2O_2-AY system was scanned in **Fig. 1**. Results drawn from **Fig. 1** were that AY could exit strong and stable RTP signal ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 471.2/644.9 nm, I_p = 182.5, curve 1.1') on PAM with the perturbation of Pb^{2+}. When H_2O_2 existed, the RTP of AY was quenched ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 471.5/645.3 nm, I_p = 171.6, curve 2.2'); while 1000.0 pg COL was added into AY- H_2O_2 system, the RTP of AY was quenched sharply ($\lambda_{ex}^{max}/\lambda_{em}^{max}$ = 471.0/643.2 nm, I_p = 55.0, curve 4.4') and ΔI_p of the catalytic reactionsystem was 116.6, which was 10.7 times than that of uncatalyzed reaction system (10.9). The ΔI_p was linear with the content of COL, which proved the probability for determining the content of COL with catalytic SS-RTP.

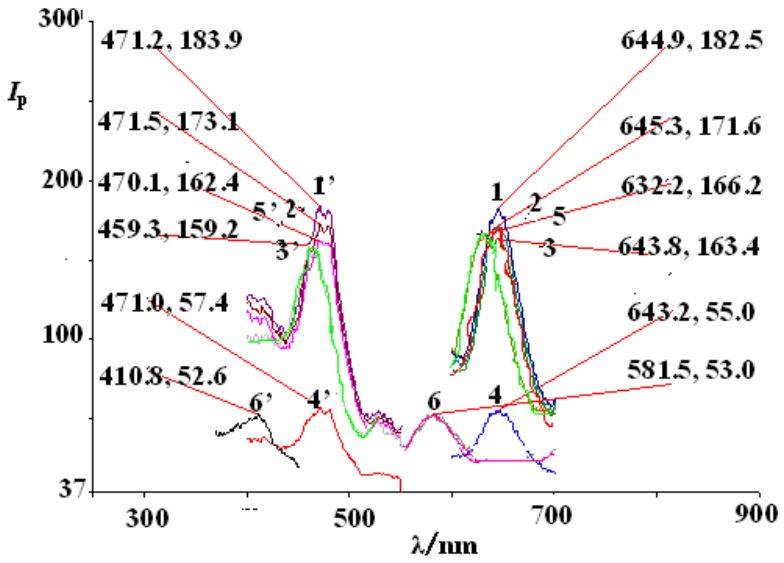


Fig. 1 RTP spectra of COL -H_2O_2-AY (Curves 1.1- 6.6 were excitation spectra, curves 1.1'-6.6' were emission spectra. The component of each curve representing is shown as following. 1.1': 6.6' + 1.00 mLAY, 2.2': 1.1' + 4.00 mL H_2O_2, 3.3': 2.2' + 10.0 pg COL, 4.4': 2.2' + 1000.0 pg COL, 5.5':1.1' + 1000.0 pg COL and 6.6': PAM. The corresponding λ_{ex}^{max} were 644.9, 645.3, 643.8, 643.2, 632.2 and 581.5, the *I*p were 182.5, 171.6, 163.4, 55.0, 166.2 and 53.0; RSDs (%) were 1.2, 1.6, 1.9, 3.5, 1.5 and 3.8, respectively)

3.3 Optimum measurement conditions

For the system containing 4.8 fg spot^{-1} COL (12.0 pg mL^{-1}), the effects of the concentration and dosage of the reagents, oxidants, solid substrates, ion perturbers, temperature and time of reaction, acidity for reaction, passing drying N_2 or not and standing time on the ΔI_p of the system were studied in a univariate approach, respectively (**Fig. 1S-11 S**).

From **Fig. 1S-11 S**, we could conclude the following rules:

1. With the increase of the concentration and dosage of AY, the ΔI_p of the system enhanced gradually. When the concentration or dosage of AY was 1.00 mL of 1.0×10^{-4} mol L^{-1}, the ΔI_p of the system reached the maximum, the reason might be that the yield of product COL'-AY reached the highest.

2. Though the ΔI_p of the system were high when $KClO_3$, $K_2S_2O_8$, KIO_4, $NaIO_4$ and $(NH_4)_2S_2O_8$ were chosen as oxidants, they were still lower than that of H_2O_2, which might result from H_2O_2 oxidation capacity reached the maximum. Further investigations found that the ΔI_p of the system enhanced

gradually with the increase of the concentration and dosage of H_2O_2. When the concentration or dosage of H_2O_2 was 4.00 mL of 2.0 %, the ΔI_p of the system reached the maximum and almost stayed invariable.

3**.** Among the three different solid substrates examined in this study, compared with paper, NCM and ACM, PAM exhibited the highest phosphorescence signal for the reason that the heavy atom Pb^{2+} solution diffused slowly on PAM, but spread rapidly when the PAM was dried [24].

4. Though the ΔI_p of the system were high when I^-, Hg^{2+} and Ag^+ were chosen as ion perturbers, they were still lower than that of Pb^{2+}. Further investigations found that the ΔI_p of the system enhanced with the increasing of concentration of Pb^{2+} and reached the maximum when 1.0 mol L^{-1} Pb^{2+} was used. Henceforth, with the further increasing of concentration of Pb^{2+}, the ΔI_p of the system decreased. The reason might be that appropriate heavy atom could increase the intersystem crossing of AY from singlet state to triplet state, which enhanced the RTP signal, while the excessive heavy atom will lead the RTP signal to quench [21].

5. The ΔI_p of the system linearly enhanced with the increasing of pH in the range of 3.30-7.00, while it reached the maximum and remained stable when the pH was in the range of 7.00-9.60. The reason might be that the catalytic reaction rate of COL reached the maximum, and the yield of product COL'-H…N-AY reached the highest.

6. As the reaction time and temperature increased, the ΔI_p of the system gradually enhanced, which might result from increasing of the catalytic ability of COL gradually. When the reaction temperature and time were 100 °C and 10 min, respectively, the ΔI_p of the system reached the maximum, which might be that the catalytic ability of COL reached the peak.

7. In this experiment, the ΔI_p of the system almost stayed invariable when drying N_2 was passed for 3-25 min. The reason might be that the effect of oxygen and humidity on the RTP was eliminated. However, as the time increasing without passing drying N_2, the ΔI_p of the system decreased, showing the quenching effect of oxygen and humidity on the RTP. Thus, the time of passing drying N_2 was 6 min.

8. The stability of RTP was the key to determine trace COL by catalytic SS-RTP. Under the optimal conditions mentioned above, the ΔI_p of the system almost stayed invariable and had good repeatability within 40 min. But the ΔI_p of the system declined gradually when the standing time was over 40 min, possibly due to the deliquescence of AY..

Results show that the ΔI_p of the system reached the maximum and remained stable when 1.00 mL of 1.0×10^{-4} mol L^{-1}AY, 4.00 mL of 2.0% H_2O_2 and 1.00 mol L^{-1} Pb^{2+} were used, reaction temperature was 100 °C, reaction time was 10 min, the time of passing drying N_2 was 6 min and standing time was within 40 min after being cooled by flowing water for 5 min, H_2O_2, PAM and Pb^{2+} were used as oxidant, solid substrate and ion perturper, respectively. Under the optimal conditions above, the pH value of reaction solution was 7.00.

3.4 Working curve, linear range, precision and detection limit

Under the optimum conditions, the ΔI_p of the system had linear relationship with the content of COL (**Fig. 2**) .The linear range, regression equation of the working curve, correlation coefficient (*r*), relative standard deviation (RSD, 0.16 and 16.0 fg $spot^{-1}$ COL were determined in parallel for 7 times), LOD (The blank solution was measured for 11 times, and the average value of I_p was 171.6. The Sb calculated was 0.083, 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) and LOQ (calculated by 10Sb/*k*) of the method were compared with those of references [5, 8]. Results are listed in **Table 1**.

Table 1 Compare with some methods for determination of COL

Method	Linear range (g mL^{-1})	Regression equation of the working curve	r	RSD (%)	LOD (g mL^{-1})	LOQ (g mL^{-1})
Catalytic SS-RTP	4.0×10^{-13}-$4.0.\times10^{-11}$	ΔI_p = 6.366 + 6.792 m_{Col} / fg $spot^{-1}$, S_b = 0.083, n = 7	0.9986	1.2-3.9	9.3×10^{-14} (0.037 fg $spot^{-1}$)	3.1×10^{-13} (0.12 fg $spot^{-1}$)
Fluorimetry [5]	2.0×10^{-7}-5.0×10^{-6}	ΔF = – 27 + 398.8 C_{Col} (μg mL^{-1})	0.9987		1.5×10^{-8}	
UFLC-MS/MS [8]	1.0×10^{-10}-1.0×10^{-6}	Y = – 6.3971 + 0.0024x (ng mL^{-1})	0.9974	3.3-3.8	5.0×10^{-11}	

The LOD of this method was lower than that of Ref. [8], showing the higher sensitivity of the catalytic SS-RTP. There are two reasons for higher sensitivity of this method. firstly, catalytic reaction of COL had an amplification effect on measure signal; secondly, the perturbation effect of an external heavy atom (Pb^{2+}) improves the molecule transition rate of AY from singlet state to triplet state, causing the ΔI_p of the system to sharply enhance.

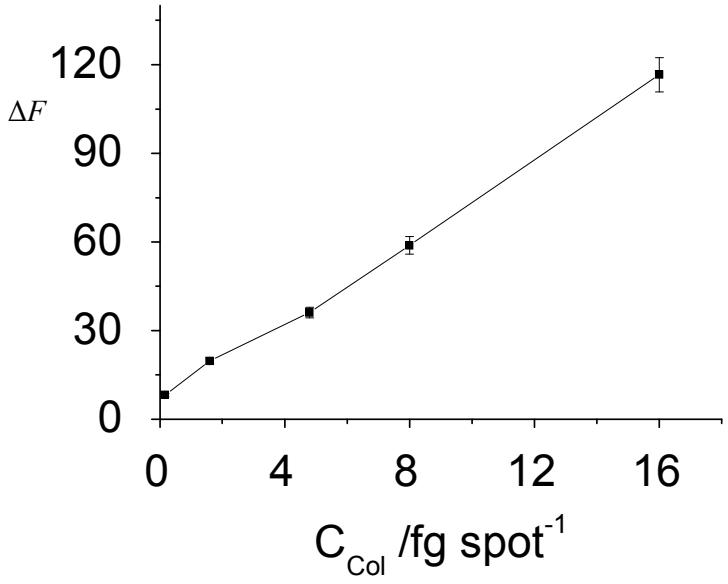


Fig. 2 Fluorescence change (ΔF) titrated with Rha under the optimum conditions

3.5 Effect on coexistence materials

Under the optimum experiment conditions described above, for the system containing 12.0 pg mL^{-1} COL and 12.0 pg mL^{-1} COL + X ng mL^{-1} coexistence (ions) materials, the allowed concentrations of coexistent ions were determined by the SS-RTP and that of Ref. [8]. When the relative error (Er) was ± 5%, the allowed concentrations of coexistent (ions) materials were compared with those in Ref. [8], and the results are listed in **Table 2S**.

Compared with Ref.[8], The allowed multiple of coexistent ions of the catalytic SS-RTP were larger than those in Ref.[8], showing that coexistent ions have little interference to the determination of COL and the catalytic SS-RTP has good selectivity. The main reason might be that the catalytic reaction had high selectivity.

3.6 Sample analysis

Six healthy volunteers had insipidity supper before taken medicine for 12 hours, fasting overnight. In the morning everyone on an empty stomach took two COL pills (1 mg) with 200 mL warm water.

According to the method in Refs.[8], 5.00 mL blood from vein of upper limb was taken after two weeks, respectively, and set in heparin tube, centrifuged at 3000 r min^{-1} centrifugal speed for 10 min. Then the supernatant of human serum was taken and was diluted to 50 mL with water, stored at -40 °C for use. Took 1.00 mL testing solution, and then the content of COL was determined by this method. Simultaneously, a standard addition recovery experiment was carried out. The results were compared with those obtained by UFLC-MS/MS [8] and are listed in **Table 2**. The significant difference analysis between catalytic SS-RTP and UFLC-MS/MS for COL detection was shown in **Table 3**.

Table 2 Results of determination of COL in serum

Sample	Catalytic SS-RTP (n = 6)					UFLC-MS/MS (n = 5)	
	Found (ng mL^{-1})	Added (ng mL^{-1})	Obtained (ng mL^{-1})	Recovery (%)	RSD (%)	Found (ng mL^{-1})	Er (%)
A	6.38	0.60	0.58	96.7	4.3	6.41	– 0.47
B	7.20	0.70	0.69	98.6	2.7	7.25	– 0.69
C	4.80	0.50	0.49	98.0	3.3	4.86	– 1.2
D	6.93	0.70	0.69	98.0	2.6	6.89	– 0.58
E	8.12	0.80	0.78	97.5	3.7	8.16	– 0.49
F	9.02	0.90	0.88	97.8	2.5	9.06	– 0.44

Table 3 Analysis of the significant differences for determination results (P = 90%, $f = n_1 + n_2 - 2 = 9$, $F_{0.90, 9} = 6.3$, $t_{0.90, 9} = 1.8$)

Sample	Catalytic SS-RTP (ng mL^{-1}, n = 6)	UFLC-MS/MS (ng mL^{-1}, n = 5)	Statistical analysis

	$\overline{X}_1$	S_1	$\overline{X}_2$	S_2	F	S	t
A	6.38	0.0306	6.41	0.0286	1.4	0.033	1.3
B	7.20	0.0532	7.25	0.0261	3.3	0.044	1.7
C	4.80	0.0605	4.86	0.0356	2.3	0.052	1.7
D	6.93	0.0418	6.89	0.0441	1.4	0.045	1.5
E	8.12	0.0691	8.16	0.0286	4.7	0.056	1.1
F	9.02	0.0460	9.06	0.0286	2.1	0.040	1.7

From **Table 2**, we can see that this method could be applied to determine COL in human serum and the results were coincident with those of UFLC-MS/MS, the recoveries of this method were 96.7 %-98.6 % and RSDs were 2.5 %-4.3%, showing higher accuracy and good precision. Besides, seen from **Table 3**, the F was 1.4, 3.3, 2.3, 1.4, 4.7 and 2.1 for the serum samples, respectively, indicating that there was no significant differences between S_1 and S_2, and the corresponding t was 1.3, 1.7, 1.7, 1.5, 1.1 and 1.7, respectively, indicating that there was also no significant differences between $\overline{X}_1$ and $\overline{X}_2$. Obviously, the catalytic SS-RTP with sensitivity and selectivity suitable for COL detection in serum samples.

4. Conclusion

In this paper, not only the feasibility of catalytic SS-RTP for COL detection was studied, but also the feasibility of this method with sensitivity and selectivity was discussed. Simultaneously, the kinetics constants of catalyze reaction and the reaction mechanism for COL detection were also studied. New method is sensitive, simple, fast, selective and accurate, which is suitable for COL detection in human serum, the results were coincident with those of UFLC-MS/MS. This study strongly promote the research progress of COL detection technology.

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