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An ultrasensitive post chemiluminescence reaction of ammonium in NBS–dichlorofluorescein system and its application

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ABSTRACT

A strong post chemiluminescence (PCL) phenomenon was observed when ammonium was injected into the reaction mixture after the finish of CL reaction of N-bromosuccinimide (NBS) and dichlorofluorescein. Based on this, a sensitive flow injection PCL method was established for the determination of ammonium. The possible CL mechanism of the reaction was proposed based on a series of experiments. The PCL intensity responded linearly to the concentration of ammonium in the range 3.0×10^{-11}–1.0×10^{-7} g mL^{-1} with a detection limit of 1×10^{-11} g mL^{-1}. The relative standard deviation (R.S.D.) was 1.4% for 1.0×10^{-9} g mL^{-1} ammonium ($n = 11$). This method had been applied to the determination of ammonium in samples of mineral water, tap water and river water.

1. Introduction

Ammonia is an important nitrogenous substance. The ammonia content in environmental water is an important indicator to evaluate the water quality [1]. Ammonia is the important indicator of cirrhosis [2], hepatic encephalopathy [3] and uremia diagnosis [4], it is also very necessary to monitor the ammonia content in the biological fluids like blood, urine and cerebrospinal fluid [5]. Furthermore, many nitrogenous organic compounds can be determined via ammonia for these compounds can be converted to ammonia [6] with the help of the corresponding enzyme.

The present methods for the determination of ammonia include UV–vis spectrometry [7,8], electrochemical method [9], ion chromatography [10], fluorescence spectrometry [11], enzymatic analysis [12] and so on. The analytical figures of some methods for ammonia determination were compared in Table 1. The detection limits of these methods are not satisfied enough. Therefore, to study new strategies of ammonia analysis with a lower detection limit are of great significance in environmental monitoring, clinical laboratory, life science research, drug analysis and food analysis.

Chemiluminescence (CL) analysis has attracted much attention in various fields for its characteristics such as high sensitivity, wide linear range and simple instruments. A few CL methods had been reported for the determination of ammonia based on the CL reaction of ammonium with hypobromite or NBS, and the sensitivity of these methods showed no significant improvement with the detection limit in the order of 10^{-9} g mL [13–19]. Although Halvatzis and Potamia [14] tried to improve the sensitivity by using dichlorofluorescein as a sensitizer, the results were not satisfied because the high background signal from NBS–dichlorofluorescein reaction under this condition cannot be eliminated. Influenced by this, the sensitivity of the CL method was decreased. Lu and co-workers [20] found that a new CL reaction will be generated when adding some substances into the solution after the former CL reaction had finished, such reaction was called post chemiluminescence (PCL) reaction. The sensitivity of the method based on this kind of PCL reaction was always improved dramatically since the background signal was almost eliminated utilizing this PCL reaction principle. In this work, a PCL reaction of ammonium in the NBS–dichlorofluorescein system was found and a new PCL method for the determination of ammonium was established combining the PCL reaction with the flow injection technique. This method was applied to the determination of ammonium in samples of mineral water, tap water and river water. This new strategy showed an important value for the microanalysis and ultratrace analysis of ammonia.

2. Experimental

2.1. Apparatus

The flow system employed in this work is shown in Fig. 1. Two peristaltic pumps (Remex Analytical Instrument Co. Ltd., Xi'an, China) were used to deliver all flow streams. Polytetrafluoroethy-

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Table 1
Comparison of different methods for the ammonium determination.

Method	Detection limit (g mL^{-1})	Linear range (g mL^{-1})	Reference
UV–vis spectrometry	3.5×10^{-7}	1.0×10^{-6}–2.0×10^{-5}	[8]
Electrochemical method	4.2×10^{-6}	1.0×10^{-5}–9.5×10^{-5}	[9]
Ion chromatography	–	1.0×10^{-7}–8.0×10^{-5}	[10]
Fluorescence spectrometry	1.2×10^{-9}	0–7.0×10^{-7}	[11]
Enzymatic method	1.3×10^{-7}	0–1.6×10^{-7}	[12]
Chemiluminescence method	3.3×10^{-7}	5.3×10^{-7}–5.3×10^{-5}	[13]
	4.5×10^{-7}	5.4×10^{-6}–5.4×10^{-7}	[14]
	2.1×10^{-8}	5.3×10^{-8}–5.3×10^{-6}	[16]
	8.0×10^{-9}	2.7×10^{-8}–7.5×10^{-7}	[17]
	1.1×10^{-9}	1.1×10^{-8}–1.1×10^{-6}	

lene (PTFE) flow tubes (0.8 mm i.d.) were used to connect all the components in the flow system. Injection was done using an eight-way injection valve equipped with a sample loop (90 μL). The flow cell was made by coiling of colorless glass tube (20 cm length, 2 mm i.d.) into a spiral disk shape and was located directly facing the window of the photomultiplier tube (PMT). The CL signal was monitored using an IFFM-D multifunction chemiluminescence analyzer (Remex Analytical Instrument Co. Ltd., Xi'an, China).

Fluorescence spectra were obtained by a 970CRT spectrofluorometer (Shanghai Analysis Instrument Main Plant, China). CL spectra were obtained by a refitted 970CRT spectrofluorometer. UV absorption spectra were measured on a UV-2550 spectrophotometer (SHIMDZU, Japan). Ultrapure water was supplied by a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

2.2. Reagents

Ammonium chloride was purchased from Paini Chemical Reagent Factory (Zhengzhou, China). Dichlorofluorescein was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). NBS was purchased from Tianjin Fuchen Chemical Reagent Factory (Tianjin, China).

The standard solution of ammonium (5.00×10^{-4} g mL^{-1}) was prepared by dissolving 0.1486 g ammonium chloride with 100 mL water and then diluting to proper concentration with water.

The stock solution (1.0×10^{-2} mol L^{-1}) of dichlorofluorescein was prepared by dissolving 2.0 g dichlorofluorescein with 25 mL NaOH (1.0 mol L^{-1}) and then diluting to 500 mL with water. The NBS solution (1.0×10^{-2} mol L^{-1}) was prepared daily by dissolving 0.18 g NBS with 100 mL water. It remained stable in two days.

All the reagents used were analytical reagent grades except for ammonium chloride which was a guaranteed reagent. Ultrapure water was used throughout the whole experiment.

2.3. Procedure

As shown in Fig. 1, flow tubes were connected with NBS solution, dichlorofluorescein solution, water and ammonium solution, respectively. NBS stream was merged with dichlorofluorescein by the Y-piece 1, the above mixture reacted completely in the mixing tube L and was injected into water by a six-way injection valve, and then mixed with ammonium solution by the Y-piece 2 to produce PCL. The ammonium concentration was quantified by the relative peak height ($\Delta I = I_{\text{sample}} - I_{\text{blank}}$).

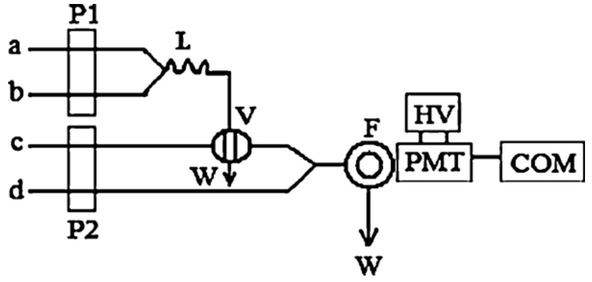

Fig. 1. Schematic diagram of the flow system. (a) NBS solution; (b) dichlorofluorescein solution; (c) H_2O; (d) ammonium standard/sample solution. P: peristaltic pump; L: mixing tube; V: six-way valve; F: flow cell; W: waste; HV: high voltage; PMT: photomultiplier tube; COM: computer.

2.4. Sample preparation

The sample solutions of tap water and river water were collected from local environment and the mineral water was purchased from local market. 0.5 mL of water sample was transferred into a 50 mL volumetric flask, then 0.5 mL of 5.0×10^{-2} mol L^{-1} HCl was added and the solution was diluted to the graduation with ultrapure water. Each water sample was treated as the above-mentioned. Then, these solutions were used as the sample solutions.

3. Results and discussion

3.1. Mechanism of the PCL reaction

3.1.1. Kinetic characteristic of the PCL reaction

The CL kinetic characteristics of the NBS–dichlorofluorescein reaction and the NBS–dichlorofluorescein–ammonium reaction were investigated using the static system of the CL analyzer. From Fig. 2, it is observed that when 1.0 mL of NBS solution (1.0×10^{-2} mol L^{-1}) was injected into 1.0 mL of dichlorofluorescein solution (5.0×10^{-4} mol L^{-1}), a CL reaction was initiated immediately (peak 1). After 50 s approximately, this CL reaction

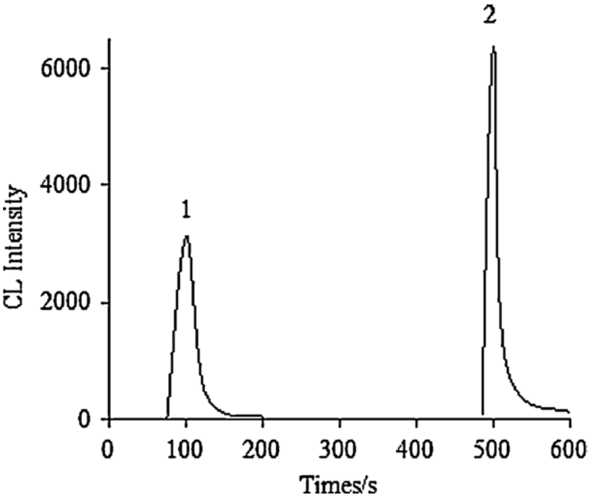

Fig. 2. Kinetic curves of the PCL reaction. (1) 1.0 mL of 1.0×10^{-2} mol L^{-1} NBS solution was injected into 1.0 mL of 5.0×10^{-4} mol L^{-1} dichlorofluorescein solution (in 0.04 mol L^{-1} NaOH); (2) 1.0 mL of 1.0×10^{-7} g mL^{-1} ammonium (in 5.0×10^{-4} mol L^{-1} HCl) was injected into the above reaction mixture.

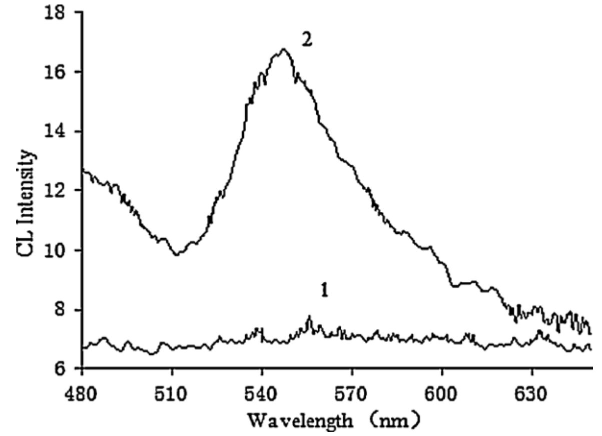

Fig. 3. CL spectra. (1) 1.0×10^{-2} mol L^{-1} NBS + 5.0×10^{-4} mol L^{-1} dichlorofluorescein (in 0.04 mol L^{-1} NaOH); (2) 1.0×10^{-2} mol L^{-1} NBS + 5.0×10^{-4} mol L^{-1} dichlorofluorescein (in 0.04 mol L^{-1} NaOH) + 5.0×10^{-5} g mL^{-1} ammonium (in 5.0×10^{-4} mol L^{-1} HCl).

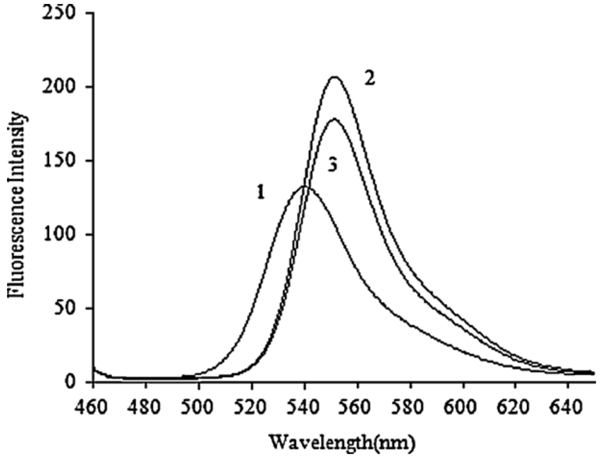

Fig. 4. Fluorescence spectra. (1) 4.0×10^{-6} mol L^{-1} dichlorofluorescein (in 4.0×10^{-3} mol L^{-1} NaOH); (2) 2.5×10^{-3} mol L^{-1} NBS + 3.0×10^{-5} mol L^{-1} dichlorofluorescein (in 4.0×10^{-3} mol L^{-1} NaOH); (3) 2.5×10^{-3} mol L^{-1} NBS + 3.0×10^{-5} mol L^{-1} dichlorofluorescein (in 4.0×10^{-3} mol L^{-1} NaOH) + 2.0×10^{-9} g mL^{-1} ammonium (in 2.5×10^{-5} mol L^{-1} HCl).

terminated and the CL signal decline to the baseline. Subsequently, a new stronger PCL reaction (peak 2) was initiated when 1.0 mL of 1.0×10^{-7} g mL^{-1} ammonium (in 5.0×10^{-4} mol L^{-1} HCl) was injected into above reaction mixture. The PCL reaction terminated and the CL signal declined to baseline after 30 s approximately.

Under the same conditions, no CL signal was detected by using the blank solution (5.0×10^{-4} mol L^{-1} HCl) instead of the ammonium solution.

The results of above experiments showed that dichlorofluorescein could react with NBS, and the reaction initiated by ammonium in the NBS–dichlorofluorescein system was a PCL reaction.

3.1.2. *Luminant of the PCL reaction*

The CL spectrum of NBS–dichlorofluorescein and the PCL spectrum of the NBS–dichlorofluorescein–ammonium were scanned using a refitted 970CRT spectrofluorometer respectively. In Fig. 3, no obvious CL peak was observed in the NBS–dichlorofluorescein system because the CL signal was so weak, while the peak of the PCL spectrum was obtained with the maximum emission wavelength at 551.6 nm.

The fluorescence spectra of dichlorofluorescein, NBS–dichlorofluorescein and NBS–dichlorofluorescein–ammonium were respectively scanned. Fig. 4 showed that the fluorescence spectra of dichlorofluorescein had a maximum wavelength at 540.2 nm. However, a new fluorescence peak with a maximum emission wavelength at 551.6 nm was observed both in the spectrum of NBS-dichlorofluorescein and NBS–dichlorofluorescein–ammonium, which suggested that a new substance had been produced. The maximum fluorescence emission wavelength (λ_{max} = 551.6 nm) of this new product was identical with the maximum PCL wavelength (λ_{max} = 551.6 nm) of the NBS–dichlorofluorescein–ammonium PCL system. Therefore, it can be concluded that the luminant of this system was not dichlorofluorescein but may be the new product (P).

To further ascertain the luminant, the UV–vis absorption spectra of different substances and their combinations were measured. From Fig. 5, it was obviously that the absorption spectrum of dichlorofluorescein (curve 1) was consistent with that of ammonium–dichlorofluorescein (curve 2), which indicated that dichlorofluorescein could not react with ammonium. Furthermore, both the spectra of NBS–dichlorofluorescein (curve 3) and NBS–dichlorofluorescein–ammonium (curve 4) had the same maximum absorption wavelength (λ_{max} = 516.2 nm) and the absorption peak of dichlorofluorescein at 503.6 nm disappeared. It can be concluded that dichlorofluorescein was completely oxidized by NBS and a new substance was produced.

The experiments mentioned above further indicated that dichlorofluorescein was oxidized completely by NBS under such condition and a same oxidation product was existed both in the NBS–dichlorofluorescein and NBS–dichlorofluorescein–ammonium system.

3.1.3. *Mechanism of the PCL reaction*

Based on the above-mentioned experimental results, a possible CL mechanism for the PCL reaction of ammonium in the NBS–dichlorofluorescein system was proposed as: NBS hydrolyzed to produce hypobromite with strong oxidizability [13]. Dichlorofluorescein was oxidized by hypobromite and produced the product at excited state. The product came back to the ground state accompanied by CL (λ_{max} = 551.6 nm). When ammonium was added into the solution of NBS–dichlorofluorescein, it was oxidized by surplus NBS to produce N_2 [21], which was probably produced in an excited state [22], and N_2^* has the ability to chemiexcite coexisting fluorophores, such as dichlorofluorescein [14]. In this research,

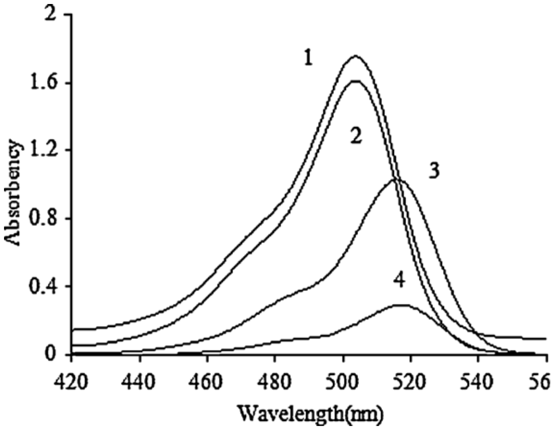

Fig. 5. UV–vis absorption spectra. (1) 3.0×10^{-5} mol L^{-1} dichlorofluorescein (in 4.0×10^{-3} mol L^{-1} NaOH); (2) 1.0×10^{-7} g mL^{-1} ammonium (in 2.5×10^{-5} mol L^{-1} HCl) + 3.0×10^{-5} mol L^{-1} dichlorofluorescein (in 4.0×10^{-3} mol L^{-1} NaOH); (3) 2.5×10^{-3} mol L^{-1} NBS + 3.0×10^{-5} mol L^{-1} dichlorofluorescein (in 4.0×10^{-3} mol L^{-1} NaOH); (4) 2.5×10^{-3} mol L^{-1} NBS + 3.0×10^{-5} mol L^{-1} dichlorofluorescein (in 4.0×10^{-3} mol L^{-1} NaOH) + 1.0×10^{-7} g mL^{-1} ammonium (in 2.5×10^{-5} mol L^{-1} HCl).

Table 2
Optimization of the reaction conditions.

Invested condition	Range of invested condition	Selected condition
Length of mixing tube	15–150 cm	90 cm
Flow rate of P_2	0.6–2.5 mL min^{-1}	1.5 mL min^{-1}
Concentration of NBS solution	5.0×10^{-3}–5.0×10^{-2} mol L^{-1}	1.0×10^{-2} mol L^{-1}
Concentration of dichlorofluorescein solution	1.0×10^{-5}–5.0×10^{-4} mol L^{-1}	5.0×10^{-4} mol L^{-1}
Concentration of NaOH solution	0.005–0.5 mol L^{-1}	0.04 mol L^{-1}
Medium of ammonium/sample solution	1.0×10^{-6}–0.1 mol L^{-1} HCl	5.0×10^{-4} mol L^{-1} HCl

$N_2{}^*$ transfer its energy to the product at ground state in the solution, the product was excitated again accompanied by PCL. The PCL signal may be due to energy transfer from the excited states of N_2 (produced by the reaction of ammonium with NBS) to the emitting species produced by the reaction of dichlorofluorescein with NBS which caused an increase in the PCL efficiency.

$$\mathrm{NBS} + \mathrm{OH^-} \rightarrow \mathrm{NHS} + \mathrm{BrO^-}$$

$$\mathrm{BrO^-} + \text{dichlorofluorescein} \rightarrow \text{product}^*(\mathrm{P}^*)$$

$$\mathrm{P}^* \rightarrow \mathrm{P} + h\nu(\lambda_{\max} = 551.6\,\mathrm{nm})$$

$$\mathrm{BrO^-} + \text{ammonium} \rightarrow \mathrm{N_2}^*$$

$$\mathrm{N_2}^* + \mathrm{P} \rightarrow \mathrm{P}^* + \mathrm{N_2}$$

$$\mathrm{P}^* \rightarrow \mathrm{P} + h\nu(\lambda_{\max} = 551.6\,\mathrm{nm})$$

The mechanism can be expressed simply as follows:

According to the report [14], dichlorofluorescein was described to be the luminant in the NBS–ammonium–dichlorofluorescein CL system and it was used as a sensitizer in the CL system. Our opinion is different from them since we believed that the luminant of this CL system was the oxidation product of NBS–dichlorofluorescein rather than dichlorofluorescein.

3.2. Optimization of the reaction conditions

A series of experiments was conducted to select the optimum analytical conditions using 5.0×10^{-9} g mL^{-1} ammonium solution. The optimized reaction conditions were summarized in Table 2.

3.2.1. Flow system

Several flow systems were designed in order to obtain the maximal PCL signal. When applying the PCL flow system without water as carrying stream, the obtained PCL signal was unstable and weak. However, when employing the system shown schematically in Fig. 1 and water as carrying stream, the reagent solution could mix adequately with sample solution as soon as they met and a strong CL signal with good precision was obtained. So, flow system in Fig. 1 was selected.

3.2.2. Length of the mixing tube

Since it was the PCL reaction of ammonium in NBS–dichlorofluorescein system that was investigated, the CL reaction between NBS and dichlorofluorescein must react adequately before initiating the new CL reaction. For this purpose, a mixing tube (L) (0.8 mm i.d.) was connected between the Y-piece and the injection valve (Fig. 1). If the mixing tube was too short, NBS and dichlorofluorescein would react deficiently and the baseline would be too high so that a low S/N (signal to noise ratio) was obtained. If the tube were too long, the PCL signal would be too weak. The length of the mixing tube was examined in the range 15–150 cm when the flow rate of each solution was fixed at 1.2 mL min^{-1}. The results showed that the maximum S/N was achieved when the length was 90 cm.

3.2.3. Flow rate of P_2

According to the kinetic curve, the PCL reaction is fast. Therefore, an appropriate flow rate is necessary to obtain a maximum CL signal. To fix the length of L at 90 cm and the flow rate of P_1 at 1.2 mL min^{-1}, the effect of the flow rate of P_2 on the PCL intensity was examined in the range 0.6–2.5 mL min^{-1}. It was found that the PCL signal increased with the increasing of the flow rate. The PCL system showed high sensitivity and precision when the flow rate of P_2 was 1.5 mL min^{-1}.

3.2.4. Concentration of NBS solution

NBS was the oxidant in the CL reaction and its concentration influences the signal of PCL. The effect of NBS concentration on the PCL intensity was examined in the range 5.0×10^{-3} to 5.0×10^{-2} mol L^{-1}. It was found that the CL signal increased with the increasing NBS concentration and reached the maximum when the concentration was 1.0×10^{-2} mol L^{-1}. The PCL intensity would be decreased if the concentration of NBS was higher. Thus, 1.0×10^{-2} mol L^{-1} of NBS solution was selected.

3.2.5. Concentration of dichlorofluorescein solution

The effect of dichlorofluorescein concentration on the PCL intensity was studied in the range 1.0×10^{-5} to 1.0×10^{-3} mol L^{-1}. The CL signal increased with the increasing dichlorofluorescein concentration in the range 1.0×10^{-5} to 5.0×10^{-4} mol L^{-1} and decreased if the concentration was higher. Finally 5.0×10^{-4} mol L^{-1} of dichlorofluorescein solution was selected.

3.2.6. Concentration of NaOH solution

The PCL reaction occurs in alkaline conditions. The alkalinity of reaction medium was controlled by varying the concentration of NaOH in dichlorofluorescein solution. The effect of NaOH concentration on the CL reaction was examined in the range 0.005–0.5 mol L^{-1}. The results showed that the suitable concentration of NaOH solution was 0.04 mol L^{-1}.

3.2.7. The medium of ammonium/sample solution

The medium of ammonium solution also affected the PCL signal. The experiment showed that PCL signal was unstable in alkaline solution and very weak in strong acidic medium. The PCL reaction occurred in a weak acidic medium and HCl solution was added in the ammonium/sample solution to adjust the acidity. The concentration of HCl was examined in the range 1.0×10^{-6} to 0.1 mol L^{-1}. The results indicated that ammonium solution con-

Table 3
The linear ranges and calibration curve.

Linear range (g mL^{-1})	Regression equation	Correlation coefficient (r)
3.0×10^{-11}–1.0×10^{-9}	$\Delta I = 42.4C(\times 10^{-10})$–7.91	0.9992
1.0×10^{-9}–1.0×10^{-8}	$\Delta I = 9.52C(\times 10^{-9}) + 25.0$	0.9990
1.0×10^{-8}–1.0×10^{-7}	$\Delta I = 57.7C(\times 10^{-8})$–42.3	0.9991

Table 4
Determination of ammonium in some water samples.

Sample	This method* (10^{-7} g/mL)	R.S.D. (%) $n = 5$	Colorimetric method* (10^{-7} g/mL)	R.S.D. (%) $n = 5$
Sample 1 (mineral water)	0.76	1.5	0.79	1.8
Sample 2 (tap water)	4.3	1.9	4.2	2.5
Sample 3 (river water)	1.5	1.3	1.6	2.1

[a]Average of five measurements.

taining 5.0×10^{-4} mol L^{-1} HCl could produce strong and steady PCL signal.

3.3. Analytical characteristics

Under the selected experimental condition, the relation between the PCL intensity and the concentration of ammonium was examined. The linear range of the method was 3.0×10^{-11} to 1.0×10^{-7} g mL^{-1}. Three calibration curves were respectively drawn in different ammonium concentration orders. The basic parameters of calibration curves were listed in Table 3.

The relative standard deviation was 1.4% for 11 independent determinations of 1.0×10^{-9} g mL^{-1} ammonium. According to the suggestions of IUPAC, the measured detection limit of the proposed method was 1×10^{-11} g mL^{-1}.

3.4. Interference study

Under the selected experimental conditions, the interference tests of some common inorganic ions and organic compounds for the determination of 1.0×10^{-9} g mL^{-1} ammonium were examined. A substance was not considered to be an interference substance within a CL signal variation of 5%. The tolerable concentration ratios of foreign species to 1.0×10^{-9} g mL^{-1} ammonium was over 1000-fold for $SO_4{}^{2-}$, $NO_3{}^{-}$, K^+, Ca^{2+}, Cu^{2+}, $NO_2{}^{-}$, Br^-, citrate, tartrate, glucose, ethanol and lactic acid; 500-fold for $CO_3{}^{2-}$, $PO_4{}^{3-}$, Cd^{2+} and Pb^{2+}; 100-fold for Zn^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Al^{3+}, Co^{2+}, acetone, lactose, and fructose; 50-fold for ascorbic acid and urea, 10-fold Cr^{3+}, Ba^{2+}; 5-fold for Ni^{2+}, Mn^{2+} and uric acid.

3.5. Sample analysis

To evaluate the applicability and reliability of the proposed method, ammonium in real samples were determined according to the method mentioned in Section 2.3. The sample solutions were prepared according to Section 2.4. The results were summarized in Table 4. The control test was performed and the result of *t*-test assumed that there was no significant difference between the results obtained by the proposed method and those obtained by the colorimetric method [23] at the confidence level of 95%.

4. Conclusion

In this paper, a PCL reaction of ammonium was found and a new method of flow injection PCL was established for the determination of ammonium in water samples. Compared with the existing methods, the proposed method has an excellent sensitivity with the detection limit of 1×10^{-11} g mL^{-1}. This work provided a promising platform for the trace and ultra-trace analysis of ammonium and some active life nitrogenous organics substances, such as amino acid, monamine, diamine, which may be transferred into ammonium through the enzymatic ammonolysis reaction.

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