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ORIGINAL PAPER

Flow-Injection Chemiluminescence Determination of Aspartic Acid in Tea Leaves Using Tris (2,2′-bipyridyl) Ruthenium (II)–Ce(IV) System

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Abstract A flow injection chemiluminescence (CL) determination of aspartic acid is described. In this work, it was observed that aspartic acid could enhance the chemiluminescence (CL) emission of $Ru(bipy)_3^{2+}$–Ce(IV) system and this enhancement effect was dependent on the concentration of aspartic acid, based on which, CL system was established for the determination of aspartic acid. Under the optimum experimental conditions, the linear range and detection limit are 2×10^{-7}–1.3×10^{-5} M and 1.5×10^{-8} M, respectively. The R.S.D. is 1.75%. (n=10). The proposed method has been applied to detect the content of aspartic acid in tea leaves with satisfactory results. The possible mechanism of the CL reaction was discussed.

Keywords Aspartic acid · Flow injection · Chemiluminescence · Ruthenium

Introduction

Tea, second to water, is one of the most widely used beverages in the world. It can be classified into six categories that are green tea, black tea, yellow tea, Oolong tea, dark compressed tea and white tea [1–6]. Comparing with them, green tea and red tea have recently gained much more scientific attention because of their medical and psychological effectiveness. Aspartic acid (Asp) (Fig. 1) is known to be one of the major constituents of tea which not only plays an important role in determining its taste and quality [7, 8]. Because of the biological and clinical importance of amino acids contained in tea, a number of approaches have been developed to determine their concentrations in different types of tea [9–10].

As the most common amino acids do not contain a chromophoric group, many of fluorescent dyes, have been used for the derivatization of amino acids. The high performance liquid chromatography (HPLC) with laser-induced fluorescence (LIF) is a common approach for the analysis of aspartic and other amino acids in tea samples in sub femtomole levels [11–15]. However, HPLC analysis has some drawbacks of long analysis time, expensive instrumentation and sample pretreatment procedures. By contrast, application of chemiluminescence method is gaining interest in analytical chemistry because it shares a number of advantages [16] including (1) low detection limits in the nanogram-or even subnanogram-per- milliliter region, (2) wide dynamic ranges (up to six orders of magnitude), (3) high signal to noise ratios resulting from the absence of a light source and the consequent absence of noise, (4) absence of Rayleigh and Raman scattering, (5) instrumental simplicity and affordability and (5) absence of toxic effects from the usual CL reagents. Tris (2,2′-bipyridyl)ruthenium(II) has been extensively used as the basis of chemiluminescence detection for a large number of compounds after oxidation to the ruthenium(III) complex [17]. The analyte reacts with the ruthenium(III) complex, and reduces it to the ruthenium(II) complex in an excited state, which then gives emission at 610 nm.

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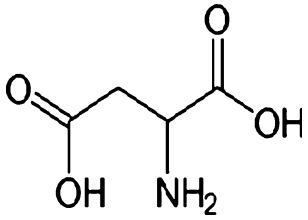
Fig. 1 Structure of aspartic acid



In this experiment, it was observed that Asp could enhance the CL emission of tris (2,2′-bipyridyl)ruthenium (II)–Ce(IV) system and the enhancement degree was linearly related to the amount of Asp added. Under the optimum experimental conditions, the CL intensity is linear to the concentration of Asp in the range of 2×10^{-7}–1.3×10^{-5} M. The limit of detection (LOD) was found to be 1.5×10^{-8} M. The relative SD for ten repeated measurements of 1×10^{-5} M aspartic acid was 1.75%. The method has been successfully applied to determine Asp in tea leaves. The reaction mechanism of the proposed method was also discussed.

Experimental

Reagents

All the solution was prepared using reagent grade chemicals and doubly distilled water was used throughout. Asp acid was purchased from Sigma (St. Louis, USA). The stock solution of aspartic acid was prepared by dissolving appropriate amount of it in water. Cerium (IV) sulfate, $Ce(SO_4)_2$ (Aldrich, USA) working solution (1×10^{-2} M) was prepared by dissolving 0.0830 g of the salt in 25 ml of 8 mM H_2SO_4. A 1×10^{-2} M stock solution of $Ru(bipy)_3Cl_2$ was prepared by dissolving a required amount of the salt in water. All working solutions were prepared by appropriate dilution with deionized water.

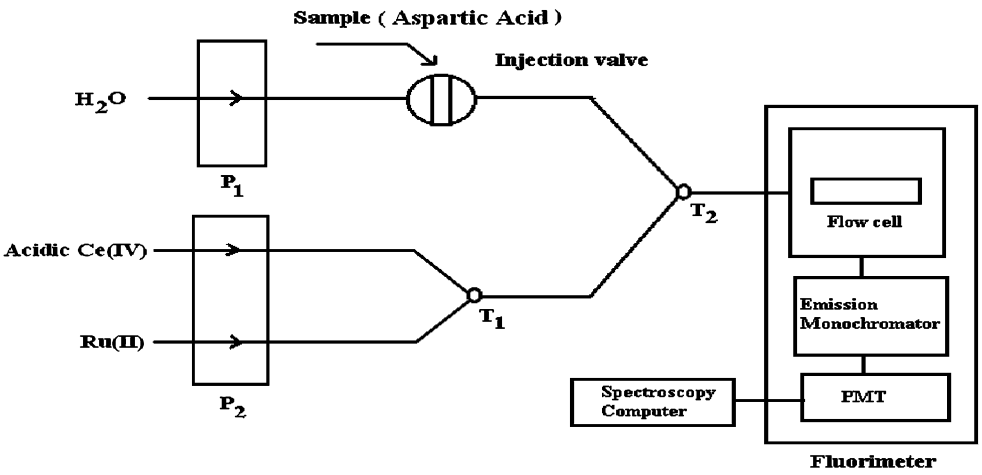
Apparatus

A schematic diagram of FIA (Flow-injection analysis) used in our work is shown in Fig. 2. A 12-channels peristaltic pump (Ismatec Model 404) with three silicon rubber tubes (1.0 mm i.d.) was used to convey all solutions. Pump P_1 delivered carrier stream (H_2O) at a flow rate of 2.5 ml min^{-1} which was incorporated with sample solution in a Rheodyne (Cotati, CA, USA) Model 7125 six-way injection valve with a loop while pump P_2 was conveyed all other CL reagents at an equal flow rate of 2.5 ml min^{-1} for each line. PTFE tubing (0.8 mm i.d) was used throughout the manifold to carry all solutions. A Spex (Edison, NJ, USA) Model FL111 spectrofluorimeter equipped with a coiled glass flow cell. (1.0 mm i.d., 20 mm total diameter) was used for detecting and recording the CL intensity of the reaction product. The data acquisition and data analysis carried out by Spex DM 3000 program. During the CL measurements, the light source of the spectrofluorimeter was switched off. The slit width of the emission monochromator was 0.25 mm. The high voltage was set to 950 V. for the photomultiplier tube (R 928, Hamamatsu, USA).

Procedure

The FIA system (Fig. 2) consisted of a three-channel manifold using two pumps. Prior to the CL measurement acquisition corresponding to the solution containing, ruthenium (II) solution stream was mixed with acidic Ce(IV) solution stream in a three-way connector, "T_1". The resulting stream was mixed with the carrier solution in the second connector, "T_2", and then reached the flow

Fig. 2 Schematic diagram of the FIA-CL manifold employed for the quantitative determination of aspartic acid. P_1, P_2: Peristaltic pumps; T_1, T_2: Y-pieces



cell in the fluorimeter, accompanying the remarkable increase of CL intensity. The full chemiluminescence intensity versus time was recorded. The increase in the CL intensity produced when a solution containing the aspartic acid was incorporated into the carrier stream, in relation to the original CL signal corresponding to a blank, was proportional to the aspartic acid concentration and was used as analytical signal. This signal was measured as peak height.

Results and discussion

Selection of the concentration of $Ru(bipy)_3^{2+}$

It is well reported that for the CL system involved in $Ru(bipy)_3^{2+}$ the emission is observed from the excited $[Ru(bipy)_3^{2+}]^*$ which is the reaction product of $Ru(bipy)_3^{3+}$ with a radical amine, therefore $Ru(bipy)_3^{2+}$ is the luminophor of the system [18]. With the solutions containing a variable amount of $Ru(bipy)_3^{2+}$ from 0.25 mM to 1.5 mM, 0.1 mM aspartic acid, 2.5 mM Ce(IV), 8.0 mM H_2SO_4 and flow-rate 2.5 ml min^{-1} the effect of the concentrations of $Ru(bipy)_3^{2+}$ on the system was investigated by determining the CL intensity of $Ru(bipy)_3^{2+}$–Ce(IV)–aspartic acid and the results are shown in the Fig. 3. The experimental results showed that with the concentration of $Ru(bipy)_3^{2+}$ increasing, the chemiluminescence intensity increased from 0.25 to 0.75 mM. The phenomenon may result due to the rapid chemiluminescence reaction kinetics because of the increased reagent to analyte radical ratio. The CL intensity then started to fall off from 0.75 mM to 1.5 mM with substantial increase in the blank value. Therefore the optimum concentration of $Ru(bipy)_3^{2+}$ is 0.75 mM with best signal to background ratio.

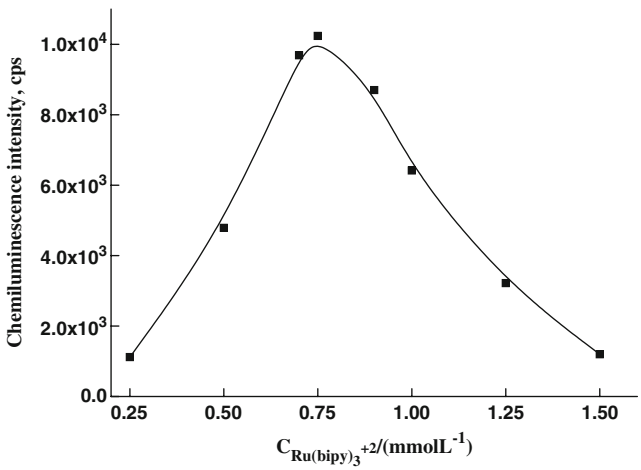


Fig. 3 The effect of the concentration of $Ru(bipy)_3^{2+}$. Conditions: 0.1 mM aspartic acid, 2.5 mM Ce(IV), 8.0 mM H_2SO_4 and flow-rate 2.5 ml min^{-1}

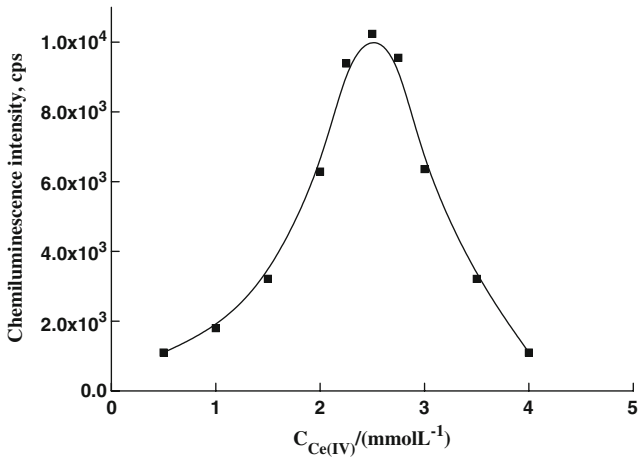


Fig. 4 The effect of the concentration of Ce(IV). Conditions: 0.1 mM aspartic acid, 0.75 mM $Ru(bipy)_3^{2+}$, 8.0 mM H_2SO_4 and flow-rate 2.5 ml min^{-1}

Optimization of Ce (IV) concentration

Ceric sulfate being a non luminescent and strong oxidizing agent [19] was utilized as the oxidant in this CL system. The effect of $Ce(SO_4)_2$ concentration on CL intensity was studied over the range 0.5 mM–4 mM $Ce(SO_4)_2$ under the optimum conditions, and the results are shown in Fig. 4. This shows that the CL intensity was increased with $Ce(SO_4)_2$ concentration when the concentration of $Ce(SO_4)_2$ was under 2.5 mM. The maximum intensity was obtained when the concentration of $Ce(SO_4)_2$ was 2.5 mM. At higher

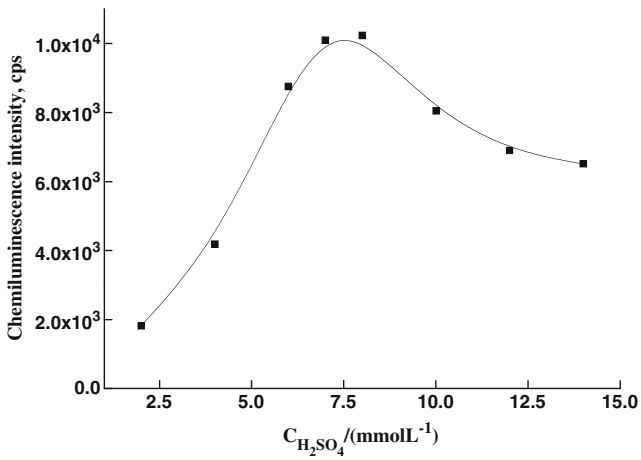


Fig. 5 The effect of the concentration of H_2SO_4. Conditions: 0.1 mM aspartic acid, 0.75 mM $Ru(bipy)_3^{2+}$, 2.5 mM Ce(IV) and flow-rate 2.5 ml min^{-1}

$Ce(SO_4)_2$ concentrations, the CL intensity decreased, which might be due to the effect of the color of the $Ce(SO_4)_2$ solution and the scattering of light emitted by the unsolvable hydrolysis product of Ce(IV) in acidic media. For this reason, an optimum point was selected at 2.5 mM $Ce(SO_4)_2$. The blank CL signal was independent of the Ce $(SO_4)_2$ concentrations.

Effect of H_2SO_4 concentration on the CL intensity

The chemiluminescence intensity depends on the concentration of H_2SO_4. The experiment was performed in the range of 2 to 14 mM H_2SO_4 under the standard conditions mentioned. The maximum intensity reached at 8 mM H_2SO_4. When the H_2SO_4 concentration was above this level, the light intensity started to decrease up to 14 mM H_2SO_4 (Fig. 5). In the range of the used H_2SO_4 concentration, the Ce(IV) species exist as sulfated complexes, such as, $Ce(SO_4)^{2+}$, $Ce(OH)(SO_4)^{1+}$, $Ce(SO_4)_2$, $Ce(SO4)_3^{2-}$, $HCe(SO_4)_3^{-}$, $HCe(SO_4)_4^{3-}$ and $Ce(SO_4)_4^{4-}$ [20, 21] and these species are in a series of equilibria with HSO_4^{-}. It has already pointed out that the reactive species of the oxidants are Ce(IV), $Ce(SO_4)_2$ and $HCe(SO_4)_3^{-}$ [22]. So, the reactive species of Ce(IV) decrease with increasing H_2SO_4 concentration, and the intensity decreases. Further more the rate of reaction is inversely proportional to the concentration of H_2SO_4 [23]. The reason behind the lower extent of oxidation with the increase of H_2SO_4 concentration is the less oxidizing power of Ce as SO_4^{2-} content is increased. For this reason, 8 mM H_2SO_4 solution was used in the work. It was noted that no change in blank signal was observed at varying concentration of H_2SO_4.

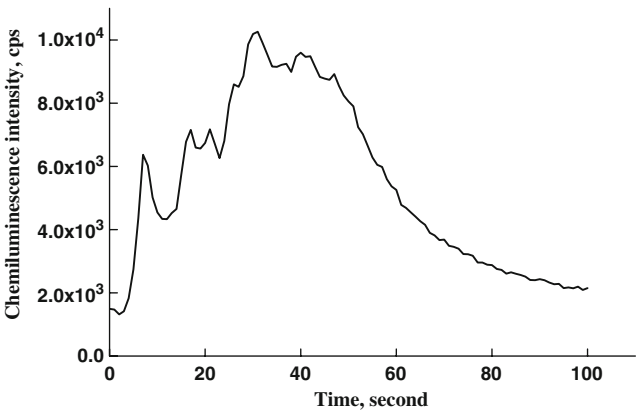


Fig. 6 The CL kinetic curve of $Ru(bipy)_3^{2+}$–aspartic acid–Ce(IV) system. Conditions: 0.1 mM aspartic acid, 0.75 mM $Ru(bipy)_3^{2+}$, 2.5 mM Ce(IV), 8.0 mM H_2SO_4 and flow-rate 2.5 ml min^{-1}

Table 1 Tolerable concentration of interfering species with respect to aspartic acid

Substances	Tolerable concentration
Tryptophan, GABA, glycine, alanine, valine, leucine, isoleucine, methionine, phenyl alanine	2.5×10^{-3} M
Catechin	1.5×10^{-3} M
Caffeine	5×10^{-3} M
Glucose, fructose, sucrose, raffinose and stachyose	1×10^{-2} M
Citric, tartaric, malic, oxalic, fumaric and succinic acid	2×10^{-5} M

Effect of flow rate on the chemiluminescence intensity

The flow rate is an important parameter in CL detection, as the time taken to transfer the excited product into the flow cell is critical for maximum collection of the emitted light [24]. In order to achieve maximum CL intensity, total flow rates in the range 1–4.5 ml min^{-1} were tested, with equal flow rates in each channel of both pumps. CL intensity increased (due to decreasing dispersion) as the flow rate increased, and reached maximum at 2.5 ml min^{-1}. Further increasing the flow rate did not change the analytical signal significantly. A low flow rate resulted in an increased residence time (>30 s). It was observed that reagent consumption increased and both peak shape and measurement rate were affected simultaneously with a higher flow rate. Therefore, a flow rate of 2.5 ml min^{-1} in each channel was selected, in consideration of greater precision and economy in the use of reagents.

Effect of sample volume on the chemiluminescence intensity

It is important to optimize the injection volume to obtain the desired sensitivity as the amounts of sample injected into the FIA system should be sufficient to permit effective CL reaction. The effect of the sample injection volume on the chemiluminescence intensity was investigated at 50, 60, 80, 100, 150, 200, 250 μL of 0.1 mM aspartic acid. The intense chemiluminescence signal and the best ratio of signal to noise were obtained when it was fixed at 150 μL. Thus, 150 μL sample solution was injected into the carrier solution.

CL kinetic curves of the systems

The CL kinetic curves of $Ru(bipy)_3^{2+}$–Ce(IV) and $Ru(bipy)_3^{2+}$–Asp–Ce(IV) systems were obtained using time base scan at the fixed emission wavelength of 610 nm. The

Table 2 Determination of aspartic acid in tea leaves and recovery results

Sample	Found ($\times10^{-6}$ M) (R.S.D %)	Recovery		
		Added ($\times10^{-6}$ M)	Found ($\times10^{-6}$ M)	Recovery± R.S.D (%)
Red tea	1.43 (3.2)	1	0.94	94±1.3
		2	1.93	96.50±3.0
		3	2.91	97±2.60
Green tea	2.31 (1.7)	1	0.95	95±3.1
		2	1.97	98.50±1.2
		3	2.92	97.33±2.2

blank signal was enhanced several times when aspartic was added to the system and the CL signal is proportional to the concentration of the aspartic acid. The residence time recorded was 30 second (Fig. 6).

Calibration curve for aspartic acid

In order to investigate the linear range of the calibration curve for aspartic acid, a series of standard solutions of Asp were added to the $Ru(bipy)_3^{2+}$–Ce(IV) system under the optimized experimental conditions: $[Ru(bipy)_3^{2+}]$= 0.75 mM, [Ce(IV)]=2.5 mM, $[H_2SO_4]$=8 mM. In common with previous reports and many similar chemiluminescence methods [25, 26], the calibration line was not linear over the whole concentration of standard solutions studied. However, the calibration approximates linearity in the range of 2×10^{-7}–1.3×10^{-5} M. The correlation coefficient of the working curve is 0.9985. The limit of detection (LOD) as defined by IUPAC, $C_{LOD}=3$ * Sb/m (where Sb is the standard deviation of the blank signals and m is the slope of the calibration graph) was found to be 1.5×10^{-8} M. The relative standard deviation for 10 repeated measurements of 1×10^{-5} M aspartic acid was 1.75%.

Interferences studies

The influence of foreign species was investigated by analyzing a standard solution of 1×10^{-5} M aspartic acid to which increasing amounts of interfering species were added. A substance was considered no interference if the variation of the CL intensity was within ±5%. The influence of some possible interfering organic compounds was investigated. The results are shown in the Table 1. Amino acids, catechin, caffeine, glucose, fructose, sucrose, raffinose and stachyose do not interfere in the determination because they are not reactive towards ruthenium–cerium CL system. Possible interference from citric, tartaric, malic, oxalic, fumaric and succinic acid can be eliminated by appropriate dilution of the sample. Therefore, the proposed method has adequate selectivity for the determination of aspartic acid in real tea leaves.

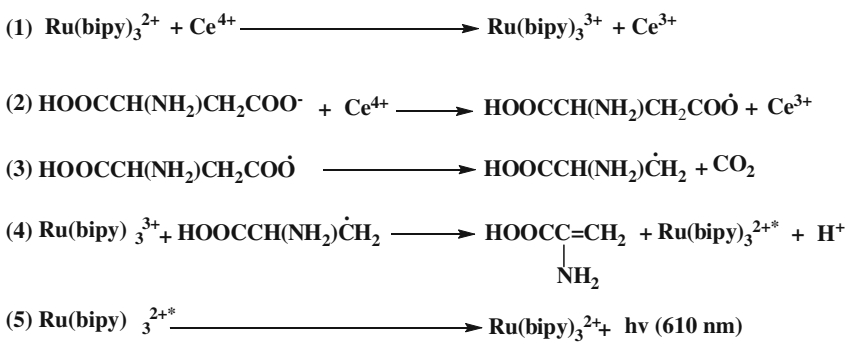
Real sample analysis

Aspartic acid was released by immersing 2 g tea leaves in 100 ml water at 50 °C and the infusion time was set at 15 min. Subsequently, the mixture was centrifuged at 3,000 rpm for 5 min at room temperature. The resulting supernatant was directly used for the analysis of aspartic acid according to the procedure. Recovery was carried out by adding different concentration of standard solutions to the sample solution. The results are shown in Table 2. As can be seen from the table the recovery results obtained by standard addition method range from 94–98.5%.

Possible mechanism of the present CL system

Rubinstein and Bard [27] reported that cerium(IV) has a higher oxidation potential than $Ru(bipy)_3^{2+}$. $Ru(bipy)_3^{2+}$ can be oxidized to $Ru(bipy)_3^{3+}$ by Ce(IV). Cerium(IV) also oxidizes the analyte to form an intermediate radical which has sufficient energy to react with $Ru(bipy)_3^{3+}$ to form $[Ru(bipy)_3^{2+}]^*$ which changes to $Ru(bipy)_3^{2+}$ with CL

Scheme 1 Oxidation of $Ru(bipy)_3^{2+}$ to $Ru(bipy)_3^{3+}$ by Ce(IV)



emission at 610 nm. The proposed mechanism is given in the Scheme 1.

Conclusions

This preliminary study shows that aspartic acid exhibits analytically useful chemiluminescence upon reaction with tris (2,2′-bipyridyl)ruthenium (II) and acidic Ce(IV). The linear range and detection limit are 2×10^{-7}–1.3×10^{-5} M and 1.5×10^{-8} M, respectively. Utilizing the method, the aspartic acid content in tea leaves can be determined with reasonable selectivity and sensitivity. The CL method proposed here is relatively simple and showed significant selectivity.

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