High-Performance Liquid Chromatographic Determination of Ascorbic Acid in Soft Drinks and Apple Juice Using Tris(2,2′-bipyridine)ruthenium(II) Electrochemiluminescence

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The electrochemiluminescence of tris(2,2′-bipyridine)ruthenium(II) [$Ru(bpy)_3^{2+}$] was applied to high-performance liquid chromatographic determination of ascorbic acid. Ascorbic acid was separated by a C_{18} reversed-phase column with a mobile phase containing 15 mM NaH_2PO_4-K_2HPO_4 (pH 6.5). The eluted ascorbic acid was mixed with 0.5 mM $Ru(bpy)_3^{2+}$ within the flow tube. The solution then passed through a thin layer flow cell equipped with a glassy carbon electrode and both ascorbic acid and $Ru(bpy)_3^{2+}$ were oxidized at +1.5 V (*vs.* Ag/AgCl). The reaction of electrolytically formed $Ru(bpy)_3^{3+}$ with oxidized ascorbic acid emitted light. The detection limit was 10 pmol for ascorbic acid at an S/N ratio of 3, and the linear calibration range was 0.06 – 80 nmol. The method was successfully applied to determination of ascorbic acid in soft drinks and apple juice.

Keywords Electrochemiluminescence, high-performance liquid chromatography, tris(2,2′-bipyridine)ruthenium(II), ascorbic acid, soft drink, apple juice

In recent years, the determination of ascorbic acid has become an important subject in the fields of biochemistry and commercial foods, because ascorbic acid play an important role in maintaining human health. The concentration of ascorbic acid affects the quality of goods, for example, preventing the degradation of soft drinks and juice or helping them retain their flavors. Many methods have been proposed for the determination of ascorbic acid. The titration and microfluorometric methods are time-consuming due to the extraction process.[1] The determination of ascorbic acid by the electrochemical method will be interfered by coexisting impurities having oxidation potentials close to that of ascorbic acid.[2] Enzymatic[3] and spectrophotometry[4,5] methods are limited by their sensitivities. Although chemiluminescent methods[6,7] have high sensitivity for the determination of ascorbic acid, the presence of an oxidizer, catalyst or peroxidase is indispensable. High-performance liquid chromatography (HPLC) offers a convenient and rapid method for automatic monitoring of ascorbic acid, so the development of a highly sensitive detector is important.

The chemiluminescence method using $Ru(bpy)_3^{2+}$ has become an attractive detection means for biochemical substances, such as amines[8] or amino acids[9], due to its low detection limits and wide linear working ranges, with relatively simple instrumentation. Hercules and Lytle[10] reported in 1966 chemiluminescence upon reduction of $Ru(bpy)_3^{3+}$ in aqueous media with hydrazine or hydroxyl ions. Chemiluminescence was also observed by Bard *et al.*[11] with some organic acids such as pyruvic, malonic or lactic, when the intermediates produced on their oxidation by Ce^{4+} reacted with $Ru(bpy)_3^{3+}$. Few reports have been found on the applications or reaction mechanisms concerning the chemiluminescence of organic acids with hydroxyl groups in the $Ru(bpy)_3^{2+}$ system.

In the course of the electrochemiluminescence (ECL) study, we found that oxidized form of ascorbic acid (ascorbate radical) produced strong luminescence in the presence of $Ru(bpy)_3^{3+}$.

This paper describes a new method for HPLC determination of ascorbic acid by chemiluminescent reaction detection. The factors influencing the ECL intensity of ascorbic acid are discussed. No significant interference was found from additives commonly found in soft drink and apple juice after a C_{18} reversed-phase column separation. This procedure is then applied to determination of ascorbic acid in those samples without any extraction or derivatization procedures.

Experimental

Chemicals and standard solution

$Ru(bpy)_3Cl_2 \cdot 6H_2O$ was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and was used without further purification. Ascorbic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All the other chemicals were of guaranteed grade. Twice-distilled deionized water was used throughout.

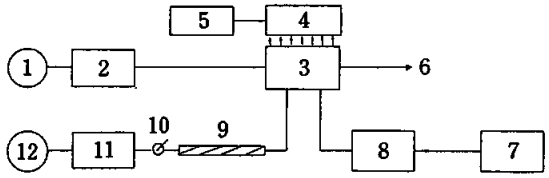


Fig. 1 Experimental setup for the ECL-HPLC. 1, reagent reservoir; 2, pump; 3, ECL cell; 4, photomultiplier tube; 5, recorder (luminescence intensity); 6, drain; 7, recorder (current); 8, potentiostat; 9, column; 10, injector; 11, pump; 12, eluent reservoir.

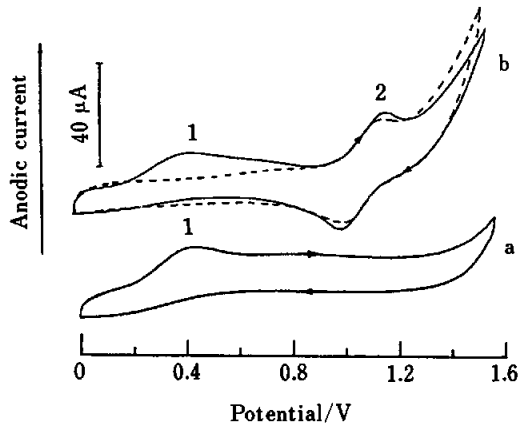


Fig. 2 Voltammetric response of CV experiment for ascorbic acid at GC electrode. a, 0.25 mM ascorbic acid+buffer; b, (---) 1 mM $Ru(bpy)_3^{2+}$+buffer, (—) 1 mM $Ru(bpy)_3^{2+}$+a; buffer, 0.2 M HAc-NaAc (pH 7.2).

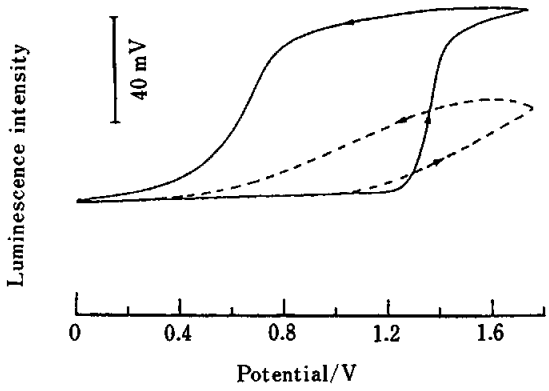


Fig. 3 ECL intensity as a function of electrode potential. (—) 1 mM $Ru(bpy)_3^{2+}$+buffer; (—) 1 mM $Ru(bpy)_3^{2+}$+buffer+ 0.25 mM ascorbic acid; buffer, 0.2 M HAc-NaAc (pH 7.8).

The stock standard solution (2 mM) of ascorbic acid was prepared with water. A working solution was prepared by dilution of the stock solution.

Apparatus

The luminescence intensity was observed by modifying a commercially available chemiluminescence analyzer (Soma Kogaku). An electrolysis cell for ECL observation was designed in our laboratory. The main body of the cell was composed of two pieces of Diflon block tightly fixed to each other. Since luminescence intensity is closely related with the orifice shape and thickness of the Teflon spacer, a spacer sheet with a thickness of 50 μm was chosen. The volume of the thin layer cell was 1.5 μl. The experimental setup is shown in Fig. 1. A three-electrode system was used for potentiostatic control of the electrolytic system. The working electrode was a glassy carbon disk (22.1 mm^2). The counter electrode set at the outlet consisted of a stainless-steel pipe, and the reference electrode was Ag/AgCl. A potentiostat (Princeton Appl. Res., type 174A) was used for electrolysis.

HPLC was performed using an LC-6A (Shimadzu, Japan) liquid chromatograph equipped with a Rheodyne 7125 sample injector (Cotati, CA, USA, 20 μl) and a $5C_{18}$ AR reversed-phase column (250×4.6 mm i.d., Waters). The mobile phase was 15 mM NaH_2PO_4-K_2HPO_4 buffer solution (pH 6.5), and the flow rate was 0.3 ml/min. The reagent solution was prepared by dissolving 0.5 mM $Ru(bpy)_3Cl_2$ in 0.2 M HAc-NaAc buffer solution (pH 7.8). The flow rate was 0.3 ml/min. A VP-6537A pen recorder (Matsushita Co. Ltd., Japan) was used for recording the luminescence intensity.

Results and Discussion

Cyclic voltammetry and parameter optimization

To determine the optimum potential for the ECL of ascorbic acid, cyclic voltammetry (CV) was performed with a glassy carbon (GC) electrode in 0.2 M HAc-NaAc buffer (pH 7.2). An irreversible anodic wave at about +0.40 V (*vs.* Ag/AgCl) could be attributed to oxidation of dehydroascorbic acid[12], no cathodic wave was observed on the potential scan in the negative direction (Fig. 2 a, peak 1). The potential at which ascorbic acid oxidation occurred depended on the pH of the buffer solution and became less positive with raising pH. Moreover, a reversible anodic wave with E_p=+1.20 V could be obtained in the presence of $Ru(bpy)_3^{2+}$ on the potential scan in the positive direction. This resulted from oxidation of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ (Fig. 2 b, peak 2). The ECL intensity (I_{ECL}) at the same GC electrode was measured as a function of the potential, as shown in Fig. 3. The intensity was low, even when the potential scan was over +1.20 V for $Ru(bpy)_3^{2+}$ solution. Addition of ascorbic acid greatly enhanced the ECL intensity. The I_{ECL} peak was obtained by scanning the applied potential to the positive direction beyond +1.40 V. It is clear that the luminescence intensity of $Ru(bpy)_3^{3+}$ increased in the presence of ascorbic acid.

In accordance with the *i*–*E* relationship, luminescence was observed when the applied potential was over +1.0 V, and increased with the increase of applied potential (Fig. 4). Since the optimum potential range

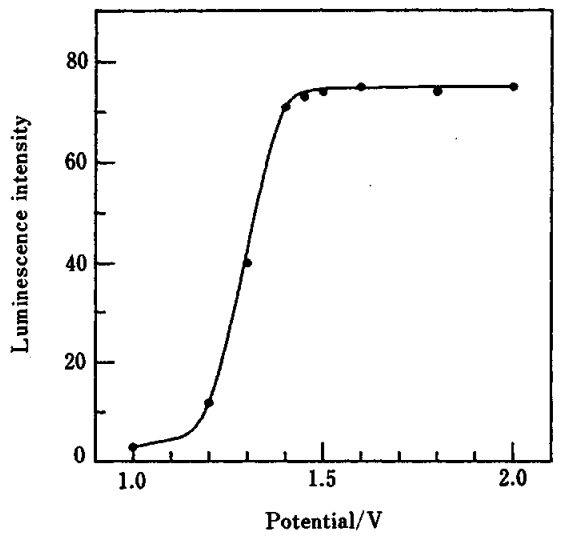


Fig. 4 Effect of applied potential on luminescence intensity. Concentration of ascorbic acid, 50 μM.

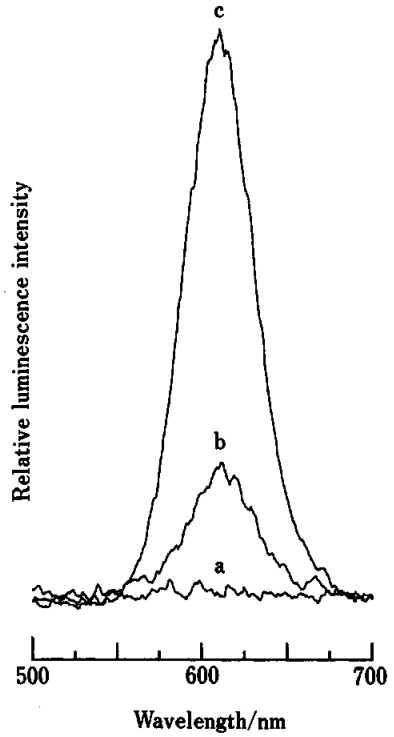
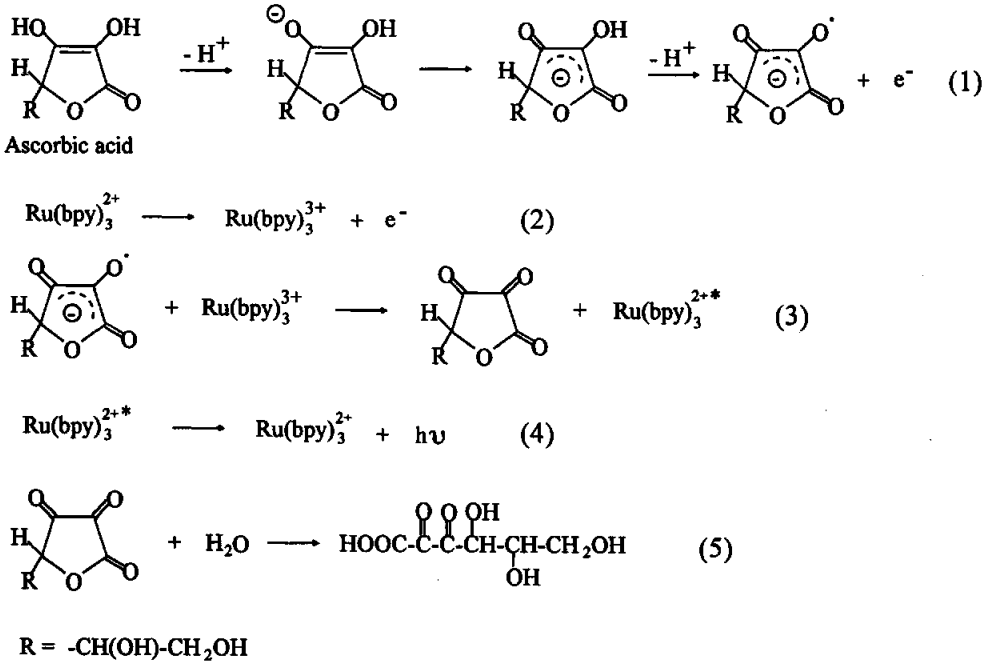


Fig. 5 ECL emission spectra. Solution, 0.2 M HAc–NaAc buffer (pH 7.2); a, 2 mM $Ru(bpy)_3^{2+}$ (potential, +0 V); b, 2 mM $Ru(bpy)_3^{2+}$ (potential, +1.5 V); c, 2 mM $Ru(bpy)_3^{2+}$+ 0.5 mM ascorbic acid (potential, +1.5 V).

for the ascorbic acid ECL was from +1.40 to +2.0 V, a potential of +1.50 V was selected for HPLC.

The chemiluminescent reaction mechanisms between $Ru(bpy)_3^{3+}$ and oxalate[14] or amino acid[15] have been studied. $Ru(bpy)_3^{2+}$ is oxidized at a GC electrode to $Ru(bpy)_3^{3+}$. At the same time, the strong reducing intermediate (radical ions) produced from oxidized oxalate or amino acid produce the excited state, $Ru(bpy)_3^{2+*}$, by an electron transfer reaction with trivalent ruthenium species. An emission having a maximum at 610 nm was produced when the excited state molecule returns to the ground state.[16]

As for our ECL system, a mechanism for the reaction



Scheme 1 The ECL reaction sequence of ascorbic acid and $Ru(bpy)_3^{2+}$.

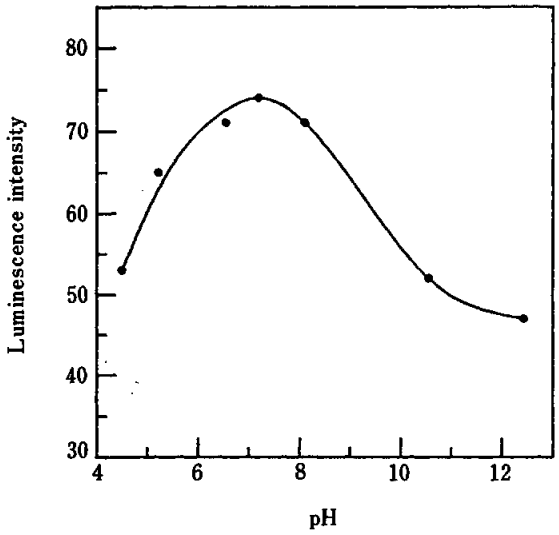


Fig. 6 Relationship between pH and luminescence intensity. Concentration of ascorbic acid, 50 μM.

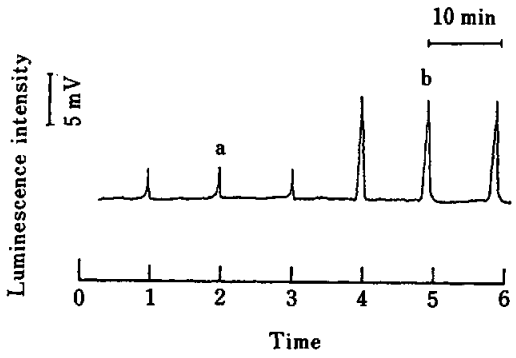


Fig. 7 Chromatograms for ascorbic acid. a, 2 μM; b, 6 μM. Eluent: 0.015 M NaH_2PO_4–K_2HPO_4, pH 6.5, flow rate, 0.3 ml/min; carrier solution, 0.5 mM $Ru(bpy)_3^{2+}$, 0.2 M NaAc; flow rate, 0.3 ml/min; potential, +1.5 V (*vs.* Ag/AgCl), glassy carbon working electrode 22.1 mm^2; separation column, $5C_{18}AR$ (250×4.6 mm); sample volume, 20 μl, temperature, 25°C.

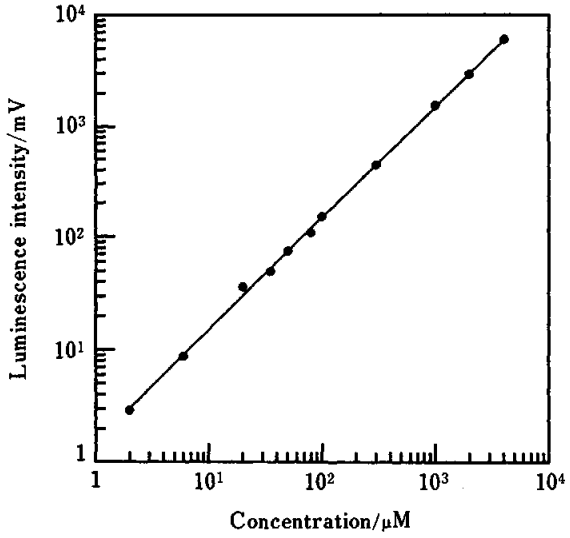


Fig. 8 The calibration curve of ascorbic acid. Caption and experiment conditions are the same as Fig. 7.

of $Ru(bpy)_3^{3+}$ with the oxidized species of ascorbic acid is proposed in Scheme 1. It is based on the fact that ascorbate radical will be formed by the oxidation of ascorbic acid at the GC electrode (1)[17], and will then reacts with $Ru(bpy)_3^{3+}$ (3) to yield luminescence (4). The electrochemical and ECL behavior of ascorbic acid and $Ru(bpy)_3^{2+}$ in Figs. 2 and 3 supports our reasoning. To further confirm this mechanism, the ECL emission spectra of $Ru(bpy)_3^{2+}$ (Fig. 5b) and of a mixture of $Ru(bpy)_3^{2+}$ with ascorbic acid (Fig. 5c) were taken. The maximum emission wavelength at about 608 nm confirmed that the luminescence in the mixture of $Ru(bpy)_3^{2+}$ with ascorbic acid is due to the presence of an excited state of $Ru(bpy)_3^{2+*}$.

Carrier solution

Rubinstein and Bard[11] reported that chemiluminescence intensity due to the reaction of oxalate with $Ru(bpy)_3^{2+}$ depends on the pH of the solution, with a maximum signal at pH 6.0. Lechien *et al.*[13] pointed out that an acidic medium is suitable for ascorbic acid detection, as ascorbic acid is unstable and can be easily oxidized by the dissolved oxygen present as impurity in the alkaline solution. Our experimental results showed that ECL intensity increased with raising pH of the carrier solution over pH 4.5, and attained a constant value above pH 7.0. The luminescence intensity decreased again over pH 8.1 (Fig. 6). The suitable pH for carrier solution was thus about 7.2. The influence of the oxidation by oxygen on the ECL of ascorbic acid could be neglected as the luminescence was produced by the reaction between oxidized ascorbic acid and $Ru(bpy)_3^{3+}$ according to CV experiments.

The concentration of $Ru(bpy)_3^{2+}$ affected the luminescence intensity of ascorbic acid. Although a higher concentration of $Ru(bpy)_3^{2+}$ showed a larger response, it also yielded more noise, and hence 0.5 mM $Ru(bpy)_3^{2+}$ was accepted. ECL responses also depended on the flow rate of carrier solution. The luminescence intensity decreased with an increase in the flow rate over 0.35 ml/min, since the reaction of oxidized ascorbic acid with $Ru(bpy)_3^{3+}$ occurred as a result of the electrode reaction. The luminescence intensity increased at lower flow rates, but the blank response also increased. Thus, an appropriate flow rate was 0.3 ml/min. In addition, with the increase in the concentration of sodium acetate in carrier solution, the luminescent intensity increased until a concentration range up to 0.15 M. This is probably due to the effect on the electrolytic efficiency, and thus 0.2 M sodium acetate was used in the following experiments.

Table 1 ECL intensities of substances examined by use of an eluent containing 15 mM phosphate and 0.1 mM Bu_4NBF_4

Compound	ECL intensity/mV[a]	Retention time/min
Ascorbic acid	2890	9.89
Fructose	2.8	8.14
Glucose	0.8	8.35
Mannose	11.2	8.85
Sucrose	0.6	8.81
Ethanol	4.4	12.75
Glycerol	1.6	8.50
Mannitol	1.3	8.74
Glucitol	14.4	6.72
Retinoic acid (vitamin A)	—	—
Thiamine·HCl (vitamin B_1)	6.4	10.78
Riboflavin (vitamin B_2)	4.8	10.54
Pyridoxine·HCl (vitamin B_6)	8.0	12.54
Calciferol (vitamin D_2)	—	—
Tocopherol (vitamin E)	—	—
Saccharic acid	18.4	10.73
Benzoic acid	—	—
Maleic acid	—	—
Malic acid	5.6	8.8
Citric acid	192	13.15
Tartaric acid	5.8	12.53
Salicylic acid	—	—
Succinic acid	—	—
Tannic acid	—	—
Histidine	165	19.52
Hydroxyproline	326	8.43
Proline	359	8.78
Starch (soluble 1%)	68	7.92
Sodium oxalate (2 μM)	32	8.58
Calcium lactate	—	—
Caffeine	5.8	8.09
Sodium metabisulfite	—	—
Caramel	—	—
Yellow No. 4	—	—
Yellow No. 5 (tartrazine)	—	—
Ponceau 4R	—	—
Orange G	—	—
Red No. 102	—	—
Bule No. 1	—	—

a. At 2 mM; injection volume, 20 μl.

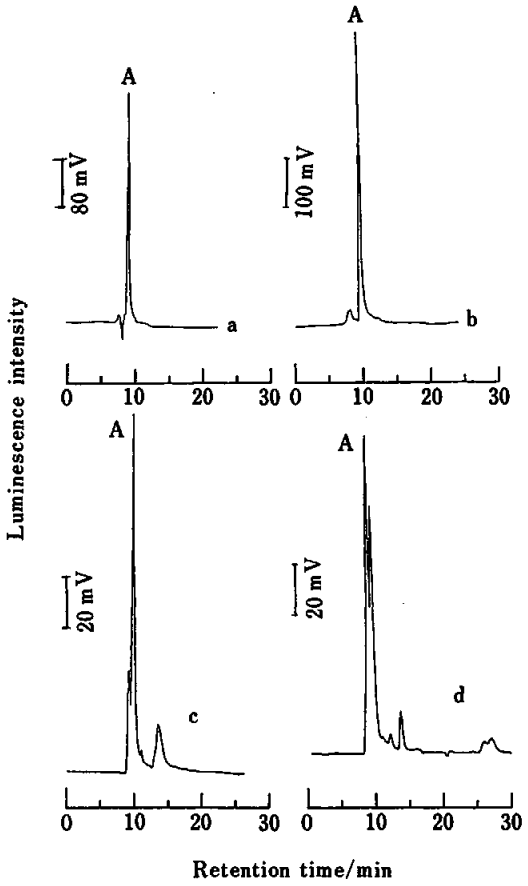


Fig. 9 Typical chromatogram for detection of soft drinks and apple juice. A, ascorbic acid; a, vitamin-C-drink A; b, vitamin-C-drink B; c, vitamin-C-drink C; d, apple juice (100%). Eluent: 0.015 M NaH_2PO_4–K_2HPO_4, $+10^{-4}$ M Bu_4NBF_4 (pH 6.5). Other conditions are the same as in Fig. 7.

HPLC analysis

For the separation of ascorbic acid, a C_{18} reversed-phase column was chosen. Typical chromatograms of ascorbic acid of 2 μM and 6 μM are shown in Fig. 7. The best separation was achieved by eluting with 15 mM phosphate buffer (pH 6.5) and a flow rate of 0.3 ml/min. Under these conditions, the calibration curve for ascorbic acid was linear from 10 pmol to 80 nmol with a slope of 1.47 mV/μM (Fig. 8). The reproducibility of the luminescence intensity of chromatograms at 50 μM was 1.5% (n=5), and the detection limit was 2 pmol at S/N 3.

Determination of ascorbic acid

This method was applied to the determination of ascorbic acid in beverages. Interferences from food additives commonly found in soft drinks and fruit juice were investigated. It is known that compounds having an –N– group, such as amines and amino acids, emit light when they react with $Ru(bpy)_3^{3+}$ in an alkaline solution.[8,9] In these interferent compounds, the ECL of vitamin B (B_1, B_2, B_6) could be observed. Among twenty of amino acids, only the secondary amino acids such as histidine, proline and hydroxyproline gave a stronger light emission. Weaker luminescence of carbohydrates and acids with –OH groups as saccharic acid, malic acid, or tartaric acid was also noted. A stronger light intensity was only produced from a few compounds such as sodium oxalate, proline, hydroxyproline, histidine and citric acid at pH 7.2. Among them, only citric acid showed the same retention time as ascorbic acid when a phosphate buffer was applied. This interference could be avoided by adding 1.0×10^{-4} M tetrabutylammoniumtetrafluoroborate (Bu_4NBF_4) as an ion-pair reagent, with a retention time at 9.89 min for ascorbic acid, and at 13.15 min for citric acid. A high concentration of Bu_4NBF_4 decreased the luminescence intensity of ascorbic acid. The ECL

Table 2 Results of ascorbic acid determination in beverages (n=5)

Berverage	Nominal value/μg ml^{-1}	Found/μg ml^{-1}	Spike test		Recovery, %
			Added/μg ml^{-1}	Found/μg ml^{-1}	
Vitamin-C-drink A	952	903.5±4.8	1000	1847	94±5.2
B	2860	2799 ±4.3	2000	4859	102±3.5
C	no labeled	86.3±2.2	100	184.5	98±2.6
Apple juice (100%)	no labeled	59.4±3.8	100	162.8	105±4.5

responses and retention times for commonly existing compounds in soft drinks and fruit juice are listed in Table 1.

Typical chromatograms obtained by the analysis of soft drinks and apple juice are shown in Fig. 9. About 10 ml of soft drinks or commercial 100% apple juice was filtered on a membrane filter with 0.45 μm pore size. A sample volume of 2.0 ml vitamin-C-drink C and commercial 100% apple juice, 0.5 ml vitamin-C-drink A, or 0.25 ml vitamin-C-drink B was neutralized to about pH 7.0 with 0.1 M NaOH, and then diluted to 10 ml. A 20 μl aliquot of these diluted solutions was then chromatographed and detected. The experimental conditions for detection are described in Fig. 9. The results for ascorbic acid analysis in the samples are listed in Table 2. The recovery of the detection was 94 to 105%, and these values were nearly within the label value for vitamin-C-drinks A and B.

In conclusion, the chemiluminescence reaction of oxidized ascorbic acid with electrogenerated $Ru(bpy)_3^{3+}$ can be applied to sensitive and reproducible detection of ascorbic acid. The sensitivity and selectivity is sufficient for the determination of the ascorbic acid in soft drinks and apple juice. Our reported method is simple and rapid compared with other methods described in the introduction. We also found that carbohydrates, alcohols and organic acids with –OH groups could yield light emission by reaction with $Ru(bpy)_3^{3+}$, and their determination will broaden the applicability of the ECL detection.

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