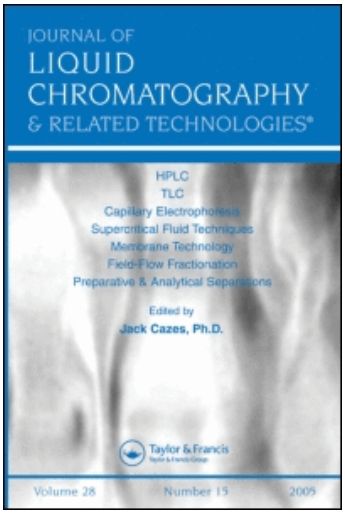

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Capillary Electrophoresis of Polyamines with Universal Indirect Chemiluminescence Detection, Using Cobalt (II) as a Probe Ion

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ABSTRACT

Highly sensitive indirect chemiluminescence detection for capillary electrophoresis (CE) of polyamines was demonstrated in this paper. In the indirect chemiluminescence (CL) detection system, a strong and stable background CL signal can be generated by the luminol-hydrogen peroxide reaction catalyzed by cobalt (II) probe ion in the running buffer. Displacement of the cobalt (II) probe ion in the running buffer, by migrating polyamine cations, results in a decrease in the background signal. Some factors affecting the separation of polyamines, such as buffer pH, buffer concentration, and additives, were systematically investigated using a commercial CE instrument with an in-house-built CL detector. Under the optimal conditions, three polyamines, putrescine (PUT),

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spermidine (SPD), and spermine (SPM) were well separated in less than 4.5 min. The detection limits of the concentration for PUT, SPD, and SPM were 1.0×10^{-8}, 5×10^{-9}, and 1.2×10^{-8} mol/L (S/N = 3), respectively. This sensitivity is about three orders better than that in the UV detection, and is almost the same as in the laser induced fluorescence detection. Our data further demonstrated that CE with indirect CL detection was a powerful tool for analysis of organic cations.

Key Words: Capillary electrophoresis; Indirect luminescence; Probe ion.

INTRODUCTION

Polyamines, such as spermidine (SPD), spermine (SPM), and their precursor, putrescine (PUT) are small polycations in neutral aqueous solution that are essential for cell viability and are present in sub-millimolar concentrations in many tissues.[1–3] Since Russell reported in 1971 that PA concentrations in the urine of some tumor patients were higher than normal,[4] the relationship between polyamines and tumor status has aroused the interest of many researchers. Traditional methods for separation of polyamines are largely confined to high performance liquid chromatography (HPLC)[5–7] and thin-layer chromatography.[8] Both methods require that the polyamines be derivatized or labeled before detection.[6,8] The derivatization and labelling procedures for polyamines are often tedious and time consuming. Capillary electrophoresis (CE) has become a powerful technique in the separation of charged biomolecules with very high resolution.[9–11] Capillary electrophoresis was superior to HPLC in terms of sensitivity and small sample size, and indicated better detection limits than gas chromatography (GC) or HPLC for polyamines analysis.[12,13] The detection methods for CE of polyamines mainly included UV,[14,15] indirect UV,[16–18] and laser-induced fluorescence detection (LIF).[12,13]

Chemiluminescence (CL) is known to be a sensitive detection method in flow injection analysis,[19,20] liquid chromatography,[21,22] and immunoassay.[23,24] Due to its simple optical system and low background nature, this method is uniquely suited to on-line detection for CE. The feasibility of using CL detection in CE has been successfully demonstrated.[25–29] Indirect CL detection was a more universal method for CE analysis of non-CL analytes.[30–32] A novel indirect CL detection for CE has been reported by our group, based on displacement of the probe ion,[31] and excellent separation efficiency and high sensitivity for analysis of 18 metal ions have been obtained. In this paper, we will explore the possibility of applying indirect CL detection for CE of polyamines using cobalt (II) as a probe ion, since they can form polycations in slight acidic solution.

EXPERIMENTAL

Reagents and Materials

Luminol was purchased from Fluka. Spermidine [SPD, $NH_2(CH_2)_3NH(CH_2)_4NH_2$], spermine [SPM, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3$], and putrescine [PUT, $NH_2(CH_2)_4NH_2$] were obtained from Sigma. Hydrogen peroxide was from Taopu Chemical Factory (Shanghai, China). Sodium acetate was a product of Guangzhou Reagents Co. (Guangzhou, China). Lactic acid and acetonitrile were obtained from Yong Hua Chemical Reagents Factory (Shanghai, China). All of the chemicals were of analytical grade or better. Water (18.0 MΩ) double-distilled and purified on the Maxima purification system (USF, ELGA), was used for the preparation of all aqueous solutions. The stock solutions of polyamines (10 mmol/L) were prepared by serial dilution of stock solutions. The sodium acetate solutions (5–20 mmol/L) were adjusted to a proper pH range from 4.0 to 5.0 with lactic acid, and 20 mmol/L sodium acetate solutions were adjusted to pH 11.80 with 1 mol/L sodium hydroxide.

Electrophoresis buffer and washing solution were filtered through 0.45 μm membrane filters prior to use.

Fused-silica capillaries with 50 μm internal diameter (i.d.) and 192 μm outer diameter (o.d.) were obtained from Polymicro Technologies Inc. (Phoenix, AZ), and fused-silica capillaries with 530 μm i.d. and 760 μm o.d. were products of Yongnian Fiber Factory (Yongnian, Hebei, China).

Capillary Electrophoresis Instrumentation

Capillary electrophoresis was performed on a commercial CE instrument (Prince Technologies, Emmn, The Netherlands) equipped with an in-house-built CL detector (as shown in [31]). Briefly, the detection interface utilized a coaxial reactor. A 44 cm capillary with 50 μm i.d. and 192 μm o.d. was used as the electrophoresis capillary, and a 12 cm capillary with 530 μm i.d. and 760 μm o.d. was used as the reaction capillary. A 1 cm detection window was made on the reaction capillary by burning off the coating. A 5.5 cm section at the end of the electrophoresis capillary was inserted into the reaction capillary, and its outlet was just positioned at the detection window. A syringe pump (MD-1001, BioAnalytical System Inc., W. Lafayette, IN) drove the reaction solutions containing hydrogen peroxide to enter the tee and flow in a sheathing profile around the electrophoresis capillary. For the CE instrument control, we used Prince software (version 4.0), for data collection Caesar software (version 4.0), both from Prince Technologies.

Capillary Electrophoresis Procedure

The new electrophoresis capillary was rinsed successively with 0.1 mol/L hydrochloric acid for 5 min, 0.1 mol/L sodium hydroxide for 2 min, water for 1 min, and electrophoresis buffer for 1 min under 1000 mPa. Sodium acetate–lactic acid solution with luminol and cobalt (II) ion was used as an electrophoresis buffer for indirect CL detection. The samples were introduced by electrokinetic injection, and electrophoresis was performed, at positive polarity, under the conditions specified in the figure legends. Luminol, dissolved in electrophoresis buffer, was driven by electroosmotic flow to migrate from the positive end to the negative end of the capillary. A syringe pump was used to deliver the reaction solution containing hydrogen peroxide. The electrophoresis capillary was first rinsed with 20 mmol/L sodium acetate (pH 11.80) for 1.5 min under 800 mPa, and then, with fresh electrophoresis buffer for 1 min under 800 mPa between each run.

RESULTS AND DISCUSSION

Indirect Chemiluminescence Detection of Polyamines

The principle of indirect CL detection we employed is different from that described in [30,32] and it is based on the displacement of probe ions in background solution by sample ions. In our indirect CL system, a trace of cobalt (II) ion, as a probe ion, was added to the electrophoresis buffer. The buffer containing cobalt (II) ion and luminol flowed from the positive end to the negative end of the capillary, driven by the electroosmotic flow and mixed with hydrogen peroxide at the detection window, and thus, CL reaction produced a strong and stable background signal. Due to the charge neutrality of the sample zone in CE, cobalt (II) ion in the electrophoresis buffer was displaced by polyamine cations when they passed the detection window. The displacement of cobalt (II) ion led to a decrease of the background signal. As a result, negative peaks were expected on the electropherograms. Figure 1 shows an electropherogram of polyamines using indirect CL detection. The positive peak denoted "s" expressed the system peak, and its migration time corresponded with that of cobalt (II) ion in direct detection. The negative peaks[1–3] were produced by displacement of cobalt (II) ion by polyamine cations. In sodium acetate–lactic acid buffer containing acetonitrile, a mixture of three polyamines was rapidly, and with high efficiency, separated within 4.5 min using CE with indirect CL detection.

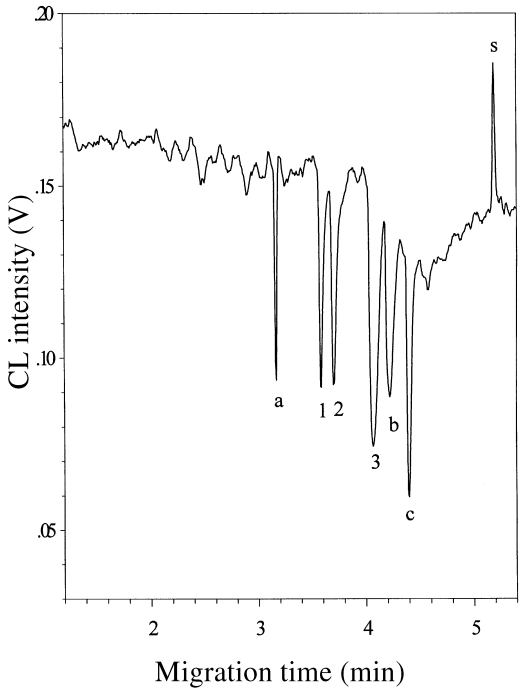

Figure 1. Indirect CL detection. of three polyamines. Twenty millimol per liter sodium acetate–lactic acid solution containing 5.0×10^{-4} mol/L luminol, 5×10^{-7} mol/L cobalt (II) ion, and 15% acetonitrile (pH 4.30) was used as the electrophoresis buffer, and 20 mmol/L sodium acetate solution covering 4 mmol/L of hydrogen peroxide was used as the reaction solution (pH = 11.80). The concentration of PUT, SPD, and SPM were 6×10^{-8}, 2×10^{-8} and 8×10^{-8} mol/L, respectively. Electrokinetic injection, 6 s at 10 kV. The temperature was ~22 (ambient), and the applied voltage was 15 kV. Peak identification: (1) PUT, (2) SPD, (3) SPM. The peak denoted "s" was a system peak, the peaks denoted "a", "b", and "c" were unknown peaks, which derived from water and reagents.

Choice of Chemiluminescence Reaction Conditions

Chemiluminescence reaction of luminol-hydrogen peroxide takes place in basic solution. Some groups[26,30] reported that the optimal pH range was from 9 to 11. In our previous work,[31] the conditions for the same CL reaction were systematically investigated based on CE. The optimal pH range for indirect CL detection of metal ions was from 11.6 to 11.8 when 20 mmol/L sodium acetate solution was used as reaction solution. Our present studies showed that the pH

range from 11.6 to 11.8 was well suited to indirect CL detection of polyamines.

Optimization of Separation Conditions

Polyamines are weak basic compounds and can form polycations in the acidic solution. The protonation degree of polyamines is associated with buffer pH, which affects the mobility of polyamines. First, we wanted to investigate the effects of a wider pH range, but, we found that luminol began precipitation when the pH of the buffer was lower than 4.0, and over the pH 5.0; the CL background signal markedly decreased, probably due to the hydrolysis of cobalt (II) ion. Therefore, in this paper, we were only able to investigate the effects of a small pH range (4.2–5.0) on the separation of polyamines, and the results obtained were shown in the Fig. 2. These data demonstrated that the migration and separation efficiency of polyamines were intensely dependent on the pH of the electrophoretic buffer. Good separation was achieved under the condition of pH 4.30. The pH effects were mainly due to the protonazition of polyamines.

Figure 3 shows the effects of the concentration of acetonitrile in the electrophoretic solution. An increase in acetonitrile concentration significantly improved separation efficiency of polyamines at the low concentrations of acetonitrile. This effect probably was attributed to the suppression of acetonitrile on the adsorption of polyamines on the capillary surface. Polyamines are protonized partially in an acidic solution to form polycations, which strongly adsorb on the inner surface of the fused silica capillary. Some organic solvents, such as methanol, ethanol, and acetonitrile, were able to suppress the absorption of the inner surface of the capillary.[33] We tried methanol, ethanol, and acetonitrile as additives and found that acetonitrile efficiently limited the absorption of the capillary and improved the separation of polyamines. Furthermore, we found that the separation efficiency of polyamines was lower when the concentration of acetonitrile was over 20% (data not shown). This phenomenon was mainly attributed to the effects of the molecular diffusion. The migration times of polyamines increased with an increase in the concentration of acetonitrile, which lead to the peak broadening of polyamines.

Figure 4 showed the influences of the buffer concentrations on the separation of polyamines. The separation efficiency dramatically improved with an increase in the concentration of electrolytes. This effect was due to the suppression of the electrolyte cations on the absorption of polyamine on the capillary. When the concentration of sodium acetate was over 20 mmol/L, the electrophoretic current markedly increased, and Joule heat-

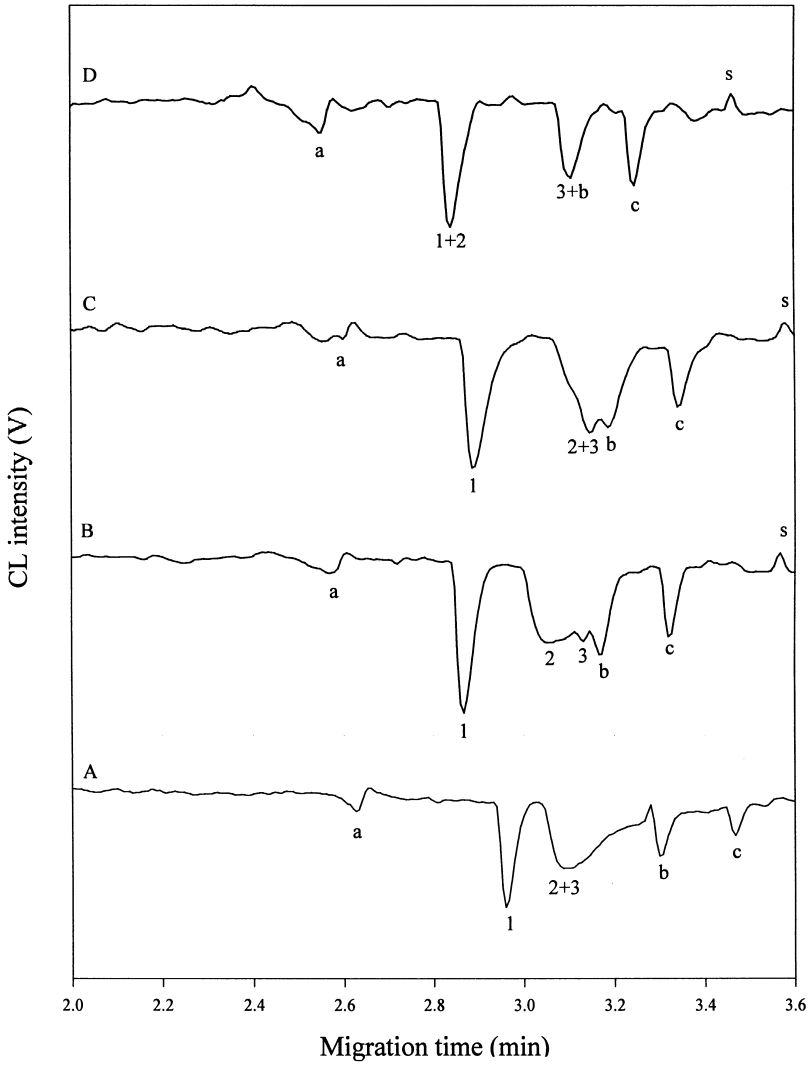

Figure 2. Effects of buffer pH on separation of polyamines. The solution of 10 mmol/L sodium acetate-lactic acid containing 5.0×10^{-4} mol/L luminol and 5.0×10^{-7} mol/L cobalt (II) ion was used as the electrophoresis buffer. The electrophoresis solution pH ranged from 4.20 to 4.60. Electropherogram identification: (A) pH 4.20, (B) pH 4.30, (C) pH 4.40, and (D) pH 4.60. The concentrations of PUT, SPD, and SPM were 2.0×10^{-7} mol/L, 8×10^{-8} mol/L, and 3.0×10^{-7} mol/L, respectively. Other conditions as in Fig. 1.

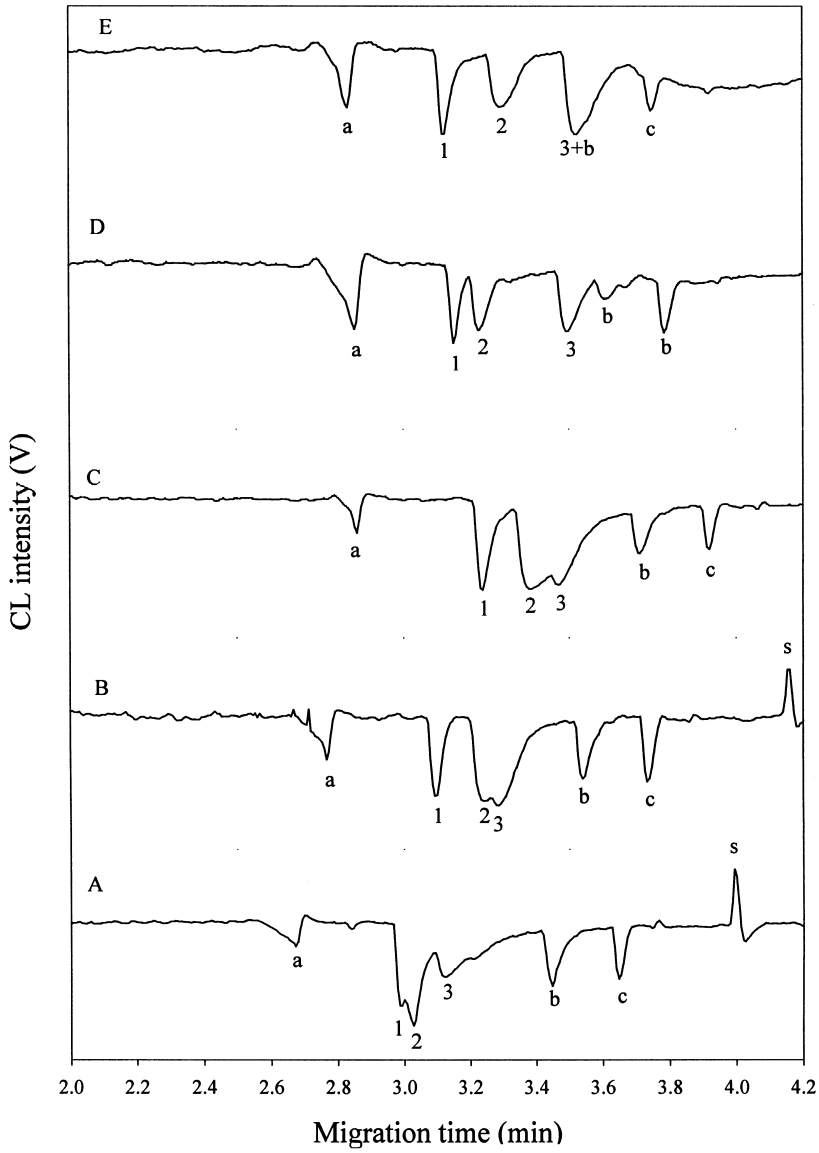

Figure 3. Effects of acetonitrile concentration on separation of polyamines. The pH of the electrophoretic buffer was 4.30. Electropherogram identification: (A) 0%, (B) 5%, (C) 10%, (D) 15%, and (E) 20%. The concentrations of PUT, SPD, and SPM were same as in Fig. 2. Other conditions as in Fig. 1.

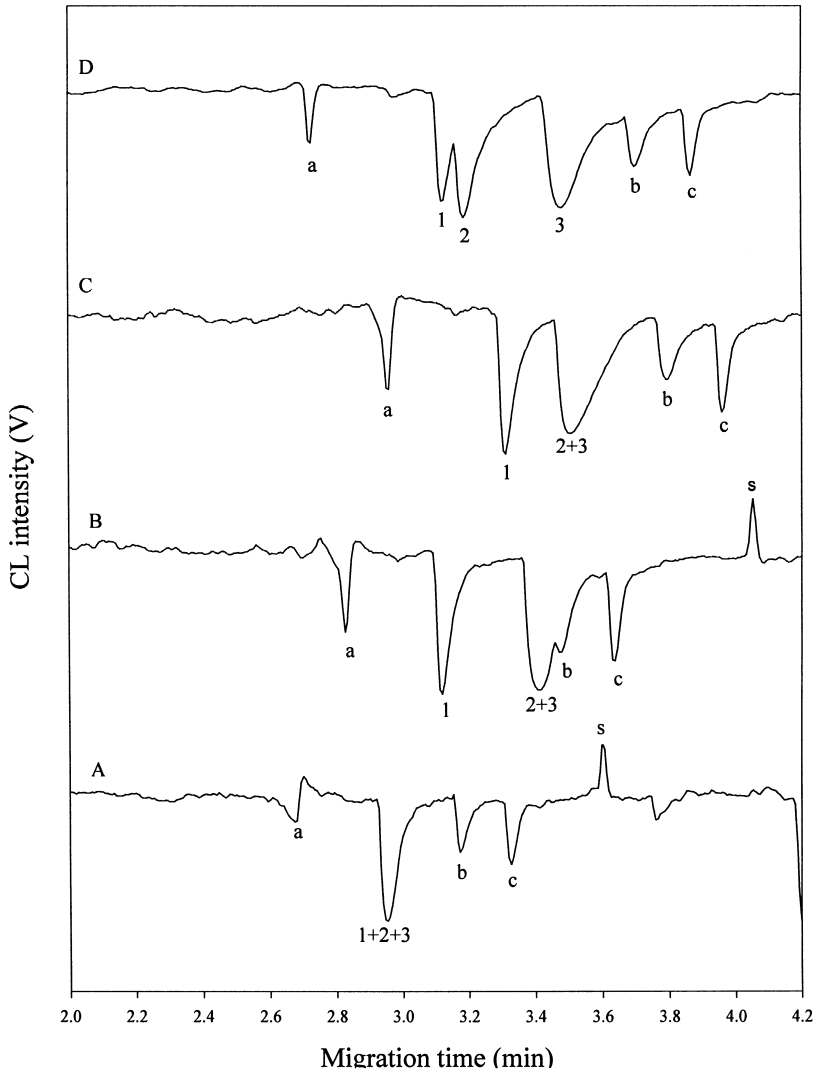

Figure 4. Effects of buffer concentration on separation of polyamines. Electropherogram identification: (A) 5 mmol/L, (B) 10 mmol/L, (C) 15 mmol/L, and (D) 20 mmol/L. The concentrations of PUT, SPD, and SPM were same as in Fig. 2. Other conditions as in Fig. 1.

Table 1. Linear ranges and a comparison of detection limits.[a]

Polyamine	Linearity (mol/L)	Detection limits (mol/L) Indirect CL	UV (14)	LIF (13)
PUT	5×10^{-7}–5×10^{-8}	1.0×10^{-8}	5×10^{-6}	6×10^{-9}
SPD	1×10^{-6}–1×10^{-7}	5×10^{-9}	2.5×10^{-5}	1.5×10^{-8}
SPM	1×10^{-6}–1×10^{-7}	1.2×10^{-8}	2.5×10^{-5}	1.3×10^{-8}

[a]The conditions of electrophoresis and reaction were the same as in Fig. 1.

ing lead to broadening of peaks. Interestingly, we examined the poor resolutions of polyamines using sodium acetate–acetic acid buffer instead of the sodium acetate–lactic acid buffer (data not shown). This reason is not clear, and was probably due to the effects of the counter ions on the migration of polyamine cations.

Detection Limits, Linearity, and Reproducibility

We measured the detection limits, linearity, and reproducibility for three polyamines using CE with indirect CL detection and the results obtained were shown in Tables 1 and 2. The detection limits of PUT, SPD, and SPM were 1.0×10^{-8} mol/L, 5×10^{-9} mol/L, and 1.2×10^{-8} mol/L (S/D = 3), respectively. Linear ranges were from 1×10^{-6} to 5×10^{-8} mol/L ($R = 0.996 - 0.999$), and relative standard deviations (RSDs) of migration times and peak areas were less than 1.2% and 9.9%, respectively. The comparison of the detection limits of several methods (Table 1) illustrated

Table 2. Reproducibility of migration times and peak areas ($n = 7$).[a]

Polyamine	Mean migration time (min)	Relative standard deviation (%) Migration time	Peak area
PUT	3.42	0.60	6.9
SPD	3.57	1.24	5.9
SPM	3.66	0.77	9.9

[a]The conditions of electrophoresis and reaction were the same as in Fig. 1.

that indirect CL was 3 orders of magnitude better than the UV and almost the same as in the LIF.

CONCLUSIONS

In this paper we described a sensitive, indirect chemiluminescence detection of polyamines based on CE using cobalt (II) as a probe ion. When compared to UV detection, this method has high sensitivity. And when compared to LIF detection, this procedure is simple and does not require derivatization of polyamines. This method may be useful for detection of polyamines in clinical diagnosis.

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