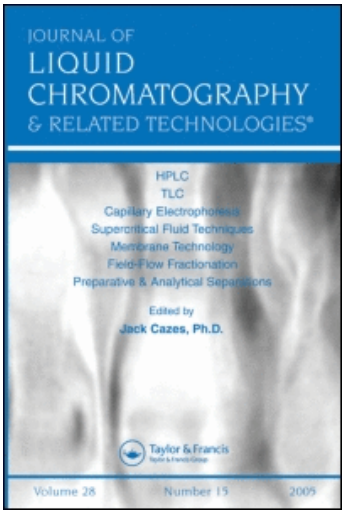

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Ming-Feng Huang[a]; Huan-Tsung Chang[a]
[a] Department of Chemistry, National Taiwan University, Taipei, Taiwan, R.O.C

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Indirect Fluorescence of Amines in Capillary Electrophoresis, Using Cresyl Violet

Ming-Feng Huang and Huan-Tsung Chang*

Department of Chemistry, National Taiwan University, Taipei, Taiwan, R.O.C.

ABSTRACT

This paper describes the analysis of amines under acidic conditions by capillary electrophoresis (CE) in conjunction with indirect laser-induced fluorescence (ILIF), using cresyl violet as a probe. In the system, a small pinhole (0.2 mm) and an interference filter (589 nm) were used to confine the light and to minimize the plasma interference from a He–Ne laser, respectively, improving the baseline stability. Adding lithium ions to the background electrolytes (BGEs) is effective to achieve narrower peak profiles, leading to better resolution. The analysis of six amines by CE–ILIF using an aqueous solution, pH 3.5, containing 5.0% methanol, 0.1 mM sulfuric acid, 0.1 mM cresyl violet, and 0.3 mM lithium was complete in 5 min, with the limits of detection (LOD) on the level of μM. Negative peak profiles are for amines with greater electrophoretic mobility than that of lithium ions, but positive peaks for the slower ones.

*Correspondence: Huan-Tsung Chang, Department of Chemistry, National Taiwan University, Roosevelt Road, Sec. 4, Taipei, 106 Taiwan, R.O.C.; E-mail: changht@ntu.edu.tw.

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To further improve the sensitivity, on-line concentration based on pH junction has been demonstrated. When injecting the sample prepared in a solution of 0.2 mM sulfuric acid, pH 3.3, at 15 kV for 60 s, and conducting the separation using the above-mentioned condition, the sensitivity improvements are greater than 10-fold compared to that injecting at 15 kV for 5 s. With the advantages of rapidity, sensitivity, and low cost, this method has proven potential for the analysis of trace amines in biological samples.

Key Words: Amines; Capillary electrophoresis; Cresyl violet; Laser-induced fluorescence; Indirect

INTRODUCTION

Indirect laser-induced fluorescence (ILIF), in conjunction with capillary electrophoresis (CE), has been tested and developed for analyzing a wide variety of solutes that have weak, or are lacking, optical properties such as fluorescence and absorption, including amines, metal ions, carboxylic acids, peptides, and carbohydrates.[1–5] With the advantages of universality, sensitivity, speed, and efficiency, CE–ILIF is a powerful tool for the analysis of small volumes of complicated biological samples, such as the determination of alkaline metal ions and anions in single cells.[6,7]

Several factors affecting the sensitivity of CE–ILIF have been revealed and related theories have been proposed.[8,9] According to Eq. (1), the dynamic reserve (DR) and transfer ratio (TR) for a system must be large in order to achieve a low detection limit (LOD).[10]

$$C_{\mathrm{LOD}} = \frac{C_{\mathrm{m}}}{\mathrm{DR} \times \mathrm{TR}} \tag{1}$$

Where C_{LOD} is the concentration limit of detection, and C_{m} is the concentration of relevant mobile-phase component, DR is the ability to measure a small change on top of a large signal, and is equal to a signal-to-noise ratio (S/N) of the background signal, and TR is the degree of displacement of the probe (co-ion) by the analyte. A large DR value is easily achieved by using a stable laser and dyes with high quantum yields. To obtain a large TR value, a fluorophore possessing a high charge density and a comparable mobility with analytes (in order to obtain symmetric peak profiles) is essential.[11] In addition, the mobility of buffer-ions should be as different from that of analytes as possible to optimize TR.[12,13]

The analysis of amines is of importance and interest because of their roles in environmental and biological processes[14–18] and has been conducted

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in CE–ILIF.[19] For optimum sensitivity and resolution, in addition to the above-addressed parameters, pH is an important parameter on the analysis of amines by CE–ILIF. Although a rapid analysis with a high resolving power can be achieved by conducting the separation at low pH, loss of sensitivity due to the competitive displacement of probes by high concentrations of hydrogen ions in the background electrolyte (BGE) and by the analytes is problematic. On the other hand, loss of resolution and poor displacement of probes by amines are disadvantageous at high pH. It has been demonstrated that the analysis of amines at pH 3.7 is proper when using quinine as a probe.[20] Unfortunately, a high-cost Ar^+ UV laser that needs expensive and routine maintenances was used in the system. In addition, loss of sensitivity due to high fluorescent background from the BGE and an unstable baseline sometimes occurs.

Our efforts to overcome these disadvantages have led to the search for fluorophores, with long maximum excitation wavelengths that can be excited by low-cost He–Ne lasers. Cresyl violet, with a maximum excitation wavelength at 592 nm and a maximum emission wavelength at 623 nm, has been chosen for this purpose. Because it possesses amino groups as shown in Fig. 1, pH should play an important role in determining its fluorescence quantum yield and mobility. In this study, we investigated the effect of pH on resolution and sensitivity. We have also found that adding lithium ions to the BGE is effective to achieve narrower peak profiles and a more stable baseline.

EXPERIMENTAL

Chemicals

All chemicals were of reagent grade and were purchased from Sigma (St. Louis, MO). Aqueous BGEs were prepared from methanol, cresyl

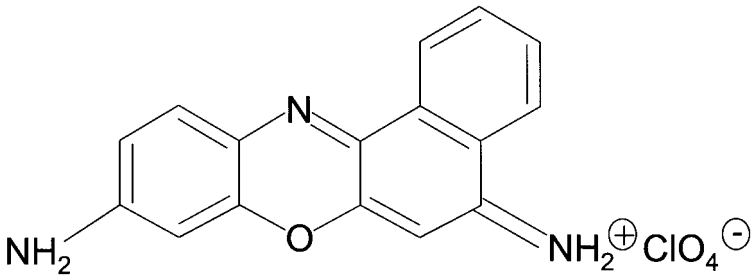

Figure 1. Structure of the probe, cresyl violet, used in this study.

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violet, sulfuric acid, and lithium, as shown in the Results and Discussion section. The stock aqueous solution of 1 mM cresyl violet was prepared in 50% (v/v) methanol. All amine samples were prepared in aqueous solutions containing 0.3 mM lithium and 0.1 or 0.2 mM sulfuric acid. An aliquot of 0.1 mL beer from Taiwan Tobacco and Liquor Corporation was diluted with 0.9 mL aqueous solution containing 0.3 mM lithium and 0.1 mM sulfuric acid.

Instrumentation

The basic design of the NACE–ILIF system has been previously described.[21] Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., Ormond Beach, FL) was used to drive electrophoresis. The entire detection system was housed in a black box with a high-voltage interlock. The high-voltage end of the separation system was put in a laboratory-made plexiglass box for safety. A 5 mW He–Ne laser with an output at 594 nm from Melles Griot (Irvine, CA) was used for excitation. Between the laser output and the focusing lens, a pinhole with a diameter of 0.2 mm and a 589 nm interference filter (Edmund Industrial Optics, Barrington, NJ) was placed to reduce the spot size and to block the plasma from the laser, respectively. The emitted light was collected with a 10× objective (numeric aperture = 0.25). One RG 610 nm cutoff filter was used to block scattered lights before the emitted light reached the photomultiplier tube (R928, Hamamatsu Photonics K. K., Shizuoka-Ken, Japan). The fluorescence signal was directly transferred through a 10-kΩ resistor to a 24-bit A/D interface at 10 Hz (Borwin, JMBS Developments, Le Fontanil, France), and stored in a personal computer. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) used were 40 cm (30 cm in effective length) × 75 μm I.D.

Separation and Calculation

Prior to use, new capillaries were equilibrated with suitable BGEs overnight. The sample injection was conducted at 15 kV for either 5 or 60 s, and the separation was carried out at 15 kV. Between runs, the capillaries were equilibrated with the BGEs for 10 min. Resolution (R_s) was calculated according to $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_1 and t_2 are the migration times, and t_{w1} and t_{w2} are the peak widths at the baseline for components 1 and 2, respectively. LOD was calculated at a signal-to-noise ratio (S/N) of 3.

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RESULTS AND DISCUSSION

Effect of the Probe and Light Source

When using cresyl violet as the probe, the analysis conducted at low pH should be efficient because it fluoresces more strongly than at high pH, leading to a high DR. For example, the ratios of the fluorescence intensities for 0.1 mM cresyl violet at pH 6.0, 4.0, and 3.0 to that at pH 8.0, are 1.4, 1.8, and 2.0, respectively. Low pH (<5.0) is also advantageous since cresyl violet is more soluble and stable (at least 4 weeks at 4°C). In addition to pH, probe concentration is another important parameter. In terms of sensitivity, high probe concentrations are not suggested according to Eq. (1). On the other hand, probe concentrations lower than 50 μM are not suitable, mainly because of low TR values due to a stronger competition of hydrogen ions to replace amines and lower DR values. To this end, the optimum concentration of cresyl violet in the BGEs is 50–100 μM under acidic conditions.

In order to monitor a small change in a highly fluorescent background, achieving a stable baseline is of considerable importance. To reach this goal, the scattering light and plasma from the He–Ne laser must be minimized. Cutting the spot size of the laser light down to about 0.2 mm by using a pinhole, makes it easier for focusing and is useful to minimize scattering light, which leads to improve the baseline stability. Adding an interference filter of 589 nm, is effective to minimize the plasma interference and further improve the baseline stability. In consequence, without using a laser stabilizer, this simple and cost-effective setup allows one to achieve a DR value of 780 when using 0.1 mM cresyl violet at pH 3.5.

Effect of Acids and Metal Ions

Compared to phosphoric acid, sulfuric acid has been found more appropriate (higher DR) to adjust the pH of the BGEs. When using phosphoric acid, relatively high concentrations were required to adjust the BGE to a certain pH value, generating high Joule heats and obtaining low TR values. Although sulfuric acid was superior, the peak profiles were still very broad and dissymmetric, which caused losses of resolution, poor sensitivity, and irreproducibility. In order to circumstance these disadvantages, co-ions such as sodium, copper, and lithium, were separately added to the BGE.[11,13,22] Adding sodium ions to the BGE, the peak profile only changed slightly, mainly because sodium ions ($\mu_{ep} = 4.91 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$) migrated faster than most of the ammonium ions; copper ions caused peak broadening, presumably due to the formation of methylamine-copper complexes and greater differential electrophoretic

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mobilities between the ammonium and copper ions. The disadvantage was overcome by adding lithium ions to the BGE, mainly due to smaller differential electrophoretic mobilities between lithium ($\mu_{ep} = 3.86 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$) and the ammonium ions (e.g., $\mu_{ep} = 3.61 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$ for diethylammonium ions). It is important to point out that the baseline also became more stable in the presence of lithium ions. Table 1 lists the effect of sulfuric acid and lithium on the peak height and bandwidth for methylamine. Although the peak profiles become narrower with increasing the concentration of lithium ions up to 2 mM, there is no clear trend in the changes in the peak height. This is because lithium ions deteriorated TR. In terms of efficiency (bandwidth) and sensitivity, the optimum condition was found when using a solution containing 0.1 mM sulfuric acid and 0.3 mM lithium ions.

Separation of Amines

Owing to very weak UV-VIS absorbance, the analysis of alkylamines by CE in conjunction with UV absorption detection is not practical. Using the

Table 1. Peak height and bandwidth for methylamine obtained under different conditions.

	Methylamine	
BGE[a]	Peak height (V)	Bandwidth (min)[b]
0.1 mM H_2SO_4/Li^+ (mM)		
0.1	43,000	0.50
0.3	40,000	0.30
0.6	16,000	0.22
1.0	12,000	0.12
0.3 mM H_2SO_4/Li^+ (mM)		
0.3	25,000	0.25
0.6	30,000	0.20
1.0	21,000	0.09
1 mM H_2SO_4/Li^+ (mM)		
0.1	24,000	0.16
0.5	17,500	0.16
1.0	5,200	0.11
2.0	7,000	0.08

[a]Separation conditions were as in Fig. 2.
[b]At the baseline.

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optimum conditions shown in Table 1, we tested the separation of six amines by CE–ILIF using cresyl violet. Figure 2 shows three negative peaks for methylamine, dimethylamine, and trimethylamine, as well as three positive peaks corresponding to diethylamine, benzylamine, and triethylamine, respectively. It is interesting to note that the negative peaks are for the amines with higher electrophoretic mobilities than that of lithium ions, indicating that cresyl violet ions were simply replaced by the cationic analytes. Compared to the first three negative peaks, the last three positive peaks that correspond to the amines with the electrophoretic mobility smaller than that of lithium ions are narrower and more symmetric. The positive peak profiles indicate that more cresyl violet ions were in the three analyte zones to balance the electricity. With more symmetric and narrower peak profiles, the sensitivity for the last three amines is greater than the first three. For example, the LOD at $S/N = 3$ for trimethylamine and triethylamine were 1.3 and 0.9 μM, respectively. The sensitivity of this method is comparable to the reported values.[14,15]

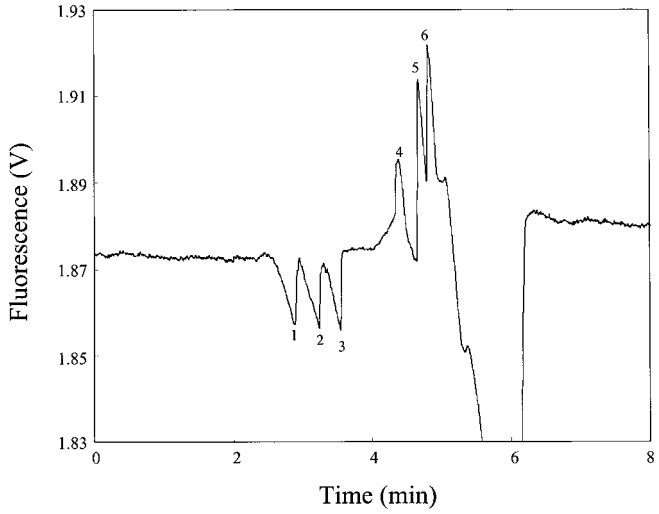

Figure 2. Electropherogram of the separation of six model amines at 15 kV by CE–ILIF using cresyl violet. The amine sample was prepared in a solution at pH 3.5 containing 0.1 mM sulfuric acid and 0.3 mM lithium. Capillary: 40 cm in total length and 30 cm in effective length; filled with a solution at pH 3.5 containing 5.0% methanol, 0.1 mM cresyl violet, 0.1 mM sulfuric acid, and 0.3 mM lithium ions. The injection was conducted at 15 kV for 5 s and the amine concentrations were all 0.1 mM. Peak identity: 1. methylamine; 2. dimethylamine; 3. trimethylamine; 4. diethylamine; 5. benzylamine; and 6. triethylamine.

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To test the feature of this method, the analysis of a diluted beer sample was conducted and the result is presented in Fig. 3. To identify some of the peaks shown in the electropherogram, the standards were spiked to the sample. Peaks 1–4 correspond to methylamine, diethylamine, benzylamine, and triethylamine, respectively. The migration times for these four analytes are slightly different from those shown in Fig. 2, mainly due to matrix effects. Our reasoning is supported by the fact that there is poor resolution and irreproducibility when injecting the beer sample without conducting dilution with the BGE. To determine the amine concentrations, standard addition was carried out, with results of 0.210 ± 0.01, 0.120 ± 0.009, and 0.060 ± 0.006 mM for diethylamine, benzylamine, and triethylamine, respectively. The other peaks in the electropherogram should correspond to different cationic solutes because the EOF (from the first system peak) is quite small (1.2×10^{-4} cm^2 V^{-1} s^{-1}) at pH 3.5. Compared to the electropherogram shown in Fig. 2, we suggest that a broad negative peak, around 3 min, should correspond to a number of cations such as metal ions (e.g., sodium, calcium and magnesium) and other amines (e.g., dimethylamine and trimethylamine) that possess greater mobility than that of lithium ion.[14–19,23,24] Several positive peaks marked as stars should correspond to amines that possess slower migration mobility than that of lithium ion,[14–19,23,24] including tyramine and histamine that have been identified by standard addition.

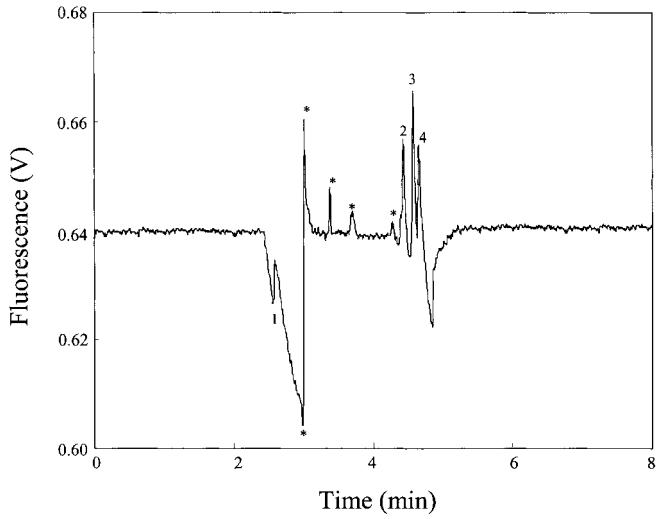

Figure 3. Separation of a beer sample diluted with the BGE by a factor of 10. Peak identity: 1. methylamine; 2. diethylamine; 3. benzylamine; and 4. triethylamine. Other conditions were as in Fig. 2. The peaks denoted by stars are reproducible but unidentified.

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Stacking

Several on-line concentration techniques in CE have been tested and developed for improving the sensitivity with varying degrees of success.[25–32] For injecting large volumes of amines, the sample should be prepared in a medium at low conductivity and low pH.[26,32] Because the concentration of lithium ions at 0.3 mM is essential in terms of sensitivity and resolution, changing the concentration of sulfuric acid was tested. The result listed in Table 2 clearly shows that the use of solutions containing high (>1 mM) or low (<10 μM) concentrations of sulfuric acid to prepare the amines was not appropriate for large-volume analysis. In the case of using high concentrations of sulfuric acid, the interference of hydrogen ion (loss of TR) is problematic. A small degree of protonation of benzylamine at high pH (0.28 at pH 4.6) is the main reason for the loss of sensitivity using the BGE containing less than 10 μM sulfuric acid. A poor stacking efficiency is another disadvantageous, which results from peak broadening because benzylamine accelerated when migrating from the sample zone (pH 4.6) to the BGE (pH 3.5). In terms of sensitivity, the optimum concentration of sulfuric acid was 0.2 mM.

When the sample was prepared in a solution at lower pH (e.g., 3.3), amines slowed down at the boundary between the sample zone and the BGE (pH 3.5). As a consequence, amines stacked when migrating from the sample zone to the BGE. The peak heights increased with increasing injection times ranging from 5 to 60 s when injecting at 15 kV. Figure 4 shows the electropherogram when injecting a sample consisting of six amines injected at 15 kV for 60 s, with a loss of resolution. The loss is mainly due to poor selectivity (shorter migration times) as the amines migrated faster at lower pH. These shortages could not be overcome by simply increasing the conductivity and pH of the BGE or decreasing the pH of samples from the standpoint of ILIF. As a result, the injection volume is limited and stacking efficiency is poor. Based on the peak height for diethylamine, the LOD is 0.4 μM, which is about a 19-fold improvement in the sensitivity when compared to that obtained in the case of injecting at 15 kV for 5 s. Although the sensitivity improvement is not great, an impressive result was shown in the LOD of 0.07 μM for triethylamine.

CONCLUSIONS

The analysis of amines under acidic conditions by CE–ILIF using cresyl violet and an orange He–Ne laser (594 nm) is presented. We have found that lithium ions play an important role in determining the peak width, the baseline stability, and TR. To the best of our knowledge, this study presents the first successful result of stacking and separating six amines by CE–ILIF based on

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Table 2. Effect of sulfuric acid and injection time on the peak height, bandwidth, and peak area for 10 μM benzylamine.

	15 kV 5 s			15 kV 60 s		
$[H_2SO_4]$ (mM)[a]	Peak height (μV)	Bandwidth (min)	Area	Peak height (μV)	Bandwidth (min)	Area
0.01	8,500	0.05	21,200	N.D.	N.D.	N.D.
0.2	14,300	0.04	36,100	48,000	0.10	3,03,400
0.5	11,000	0.03	24,200	36,100	0.08	1,84,400
1.0	10,900	0.04	22,000	N.D.	N.D.	N.D.
2.0	8,400	0.04	19,700	N.D.	N.D.	N.D.

[a]Separation conditions were as in Fig. 4.

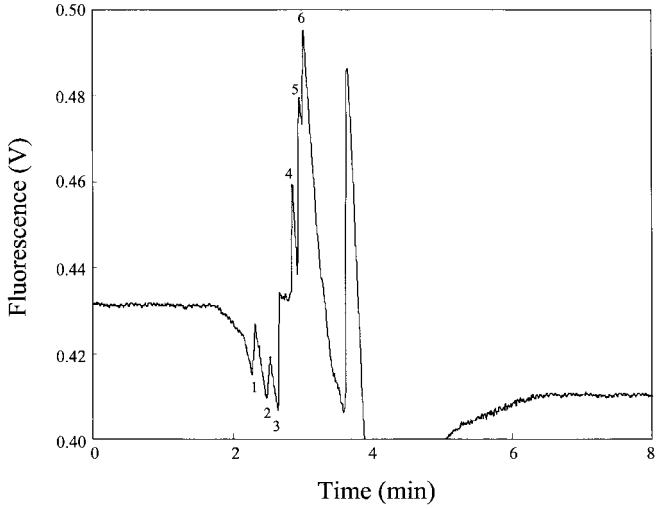

Figure 4. On-line concentration and separation of six amines at 15 kV by CE–ILIF. The amines at the concentrations of 10 μM were prepared in a buffer at pH 3.3 containing 0.2 mM sulfuric acid and 0.3 mM lithium ions. The sample injection was conducted at 15 kV for 60 s. Other conditions were as in Fig. 2.

pH junction, with the LODs down to sub μM and the sensitivity improvements up to 19-fold. With the advantages of simplicity, sensitivity, rapidness, and low cost, this method has shown great potential for the analysis of amines and cations such as peptides and metal ions.

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