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## Separation of biogenic amines by micellar electrokinetic chromatography

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### Abstract

The separation by the micellar electrokinetic method of eight biogenic amines was investigated. Optimum separation of the amines: putrescine, histamine, tyramine, cadaverine, phenethylamine, tryptamine, spermidine and spermine was obtained. The method involved extraction and derivatization of the amines with fluoresceine isothiocyanate. The electrolyte system was borate modified with sodium dodecyl sulfate. Separation was achieved in less than 15 min. Detection limit obtained with a xenon lamp-based fluorescence detector was  $10^{-8}$  M. The method developed was used for the analysis of biogenic amines in soy sauce.

*Keywords:* Derivatization, electrophoresis; Biogenic amines; Amines

### 1. Introduction

Biogenic amines are naturally occurring compounds in diverse biological systems. They are formed as a consequence of metabolic processes and by the action of microorganisms. Although some of these amines have a natural role in human physiology, large doses can lead to toxic effects and can be of special importance in patients in treatment with certain medications such as antihistamines, antimalarials and antituberculosis drugs [1]. There have been numerous cases of histamine poisoning reported to date [2]. On the other hand some biogenic amines are known to potentiate histamine toxicity [3]. The excessive level of biogenic amines is due to poor quality raw materials or poor handling. Some biogenic amines have been proposed as indicators of poor quality raw food material [4] and fish spoilage

[5]. Amines are also important in the production of carcinogenic nitrosamines after reaction with nitrites [6]. Furthermore, polyamines especially putrescine, spermidine and spermine are supposed to have an important role in normal and neoplastic cell differentiation [7], and correlation between the presence of amine for different types of cancer has been established [8].

Biogenic amines are usually analyzed by liquid chromatographic methods [9]. A review of these methods was given by Hurst [10]. Alternatively capillary electrophoresis (CE) has been used in the separation of four polyamines in rat tissue [11] but to date micellar electrokinetic chromatography has not been applied to the separation of biogenic amines. In this paper separation of some of the most common biogenic amines by CE is demonstrated. The method offers the advantage of shorter analysis time than those reported for high-performance liquid chromatography (HPLC). It is also noted that fluoresceine isothiocyanate (FITC), although not a very popular derivatizing reagent due to the long derivatization

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reaction time and time to the appearance of reagent peaks, offers the advantage of high sensitivity [12]. By using an argon-ion laser, very low detection limits have been reported [13,14]. However, the cost of such a laser is relatively high, and the wavelength range for detection is rather limited. In this work, the use of a xenon lamp-based fluorescence detector in CE for the determination of biogenic amines is demonstrated.

## 2. Experimental

### 2.1. Reagents and materials

Biogenic amine standards: Putrescine (Put); Histamine (His); Cadaverine (Cad) dihydrochloride; Tyramine (Tyr); Tryptamine (Tryp); Phenethylamine (Phe) hydrochloride; Spermidine (Spd) trihydrochloride; Spermine (Spn) tetrahydrochloride; 1,7-diaminoheptane; FITC Isomer I; sodium dodecyl sulfate (SDS) and anhydrous sodium tetraborate were purchased from Fluka (Buchs, Switzerland). Water purified with a Millipore-Q system (Millipore, Bedford, MA, USA) was used to prepare buffers for CE. Hydrogen peroxide solution (30%) was purchased from Merck (Darmstadt, Germany). Sulfuric acid was from Baker (Deventer, Netherlands). Butanol was from Ajax Chemicals (Sydney, Australia). All chemicals were of analytical reagent grade. Soy sauce sample was obtained from a local retailer.

### 2.2. Instrumentation

CE was carried out on a laboratory-built system. A Spellman power supply (Model: CZE 1000) was employed (Painview, New York, USA). A fused-silica capillary of 50 cm effective length and 50  $\mu\text{m}$  ID (Polymicro Technologies, Phoenix, AZ, USA) was used as a separation column. Peaks were detected with a Shimadzu (Kyoto, Japan) spectrofluorometer (model RF-551). The capillary was fixed in the light path by means of a polymeric tube. Data processing was performed on a Shimadzu Chromatopac CR6A integrator.

### 2.3. Procedure

Fluoresceine thiocarbamyl (FTC) amine derivatives were obtained according to the procedure first used in electrokinetic separations by Cheng and Dovichi [15]. A  $2.5 \cdot 10^{-3}$  M solution of FITC was prepared in acetone. Solutions containing  $10^{-4}$  M of each amine were prepared in 20 mM borate buffer (pH 10). One ml of each of the amine solutions was allowed to react with appropriate volumes (5  $\mu\text{l}$ , 50  $\mu\text{l}$ , 100  $\mu\text{l}$ ) of FITC solution during a period of 5 to 12 h in the dark at room temperature. Samples were diluted in the run buffer to the required concentration before injection. Capillaries were conditioned before use by the following rinsing procedure: 5 min NaOH (0.1 M); 5 min water and 10 min running buffer. Regeneration after each run was performed by rinsing the capillary with hot water for 2 min, followed by the run buffer for 2 min. When necessary, i.e. in case of irreproducible results due to adsorption, the capillary was regenerated by washing for 2 min with a solution of  $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$  (1:2), followed by hot water (10 min), 0.1 M NaOH (5 min) and running buffer (5 min). Injection was performed hydrodynamically for 10 s at a height difference of 10 cm. Applied voltage was 22 kV and the current obtained was 38  $\mu\text{A}$ .

### 2.4. Sample preparation and amine extraction

Two extraction methods were evaluated: ion-exchange extraction and liquid extraction. The ion-exchange method employed was based on that described by Saito et al. [9]. A cationic exchange resin (Amberlite GC-50) was used. The liquid extraction procedure used was based on that described by Huy and Taylor [16]. In the liquid extraction method, soy sauce samples were filtered through a 0.45  $\mu\text{m}$  nylon membrane filter. A 3 ml volume of each sample was diluted with Millipore water to 6 ml. Then 3 ml was transferred into a 15 ml capped test tube and 1 ml of 5 M NaOH was added. Anhydrous  $\text{Na}_2\text{CO}_3$  was then added to saturation. Amines were extracted 2 times with 3 ml of water-saturated *n*-butanol. The two extracts were combined and 3 ml of the aliquot was transferred to another test tube. The solution was then extracted three times with 2 ml of 0.1 M HCl, 3 ml of the acid phase was

dried under vacuum at room temperature. The dried residue was dissolved in 1 ml of 20 mM borate buffer (pH 10) and derivatized by adding 50  $\mu\text{l}$  FITC solution. Before injection 25  $\mu\text{l}$  of the internal standard solution ( $10^{-5}$  M 1,7-diaminoheptane) was added.

### 3. Results and discussion

#### 3.1. Derivatization conditions

Derivatization of amines was performed using three different amounts of fluorescent reagent solution while keeping the concentration of amines constant at  $10^{-4}$  M. Detection limits, obtained as the concentration that gives three times the noise level, are shown in Fig. 1. The results show that to maximize the sensitivity for the detection of FTC-

amines, 100  $\mu\text{l}$  of FITC should be adequate for the derivatization of the amines except for Spd. Previous studies of amino acid derivatization conditions with FITC [17,18] showed an increase in sensitivity (lower detection limits) with increasing concentration of fluorescent reagent. These results are consistent with our findings. However, at high concentrations of FITC, there were problems due to peaks of excess reagent and other side products that interfered in the identification and quantification of analytes. In addition, it was found that FTC-amines were less stable when large amounts of derivatizing reagent were used.

#### 3.2. Separation conditions

Preliminary experiments showed that without addition of SDS the resolution of the FTC-amines was

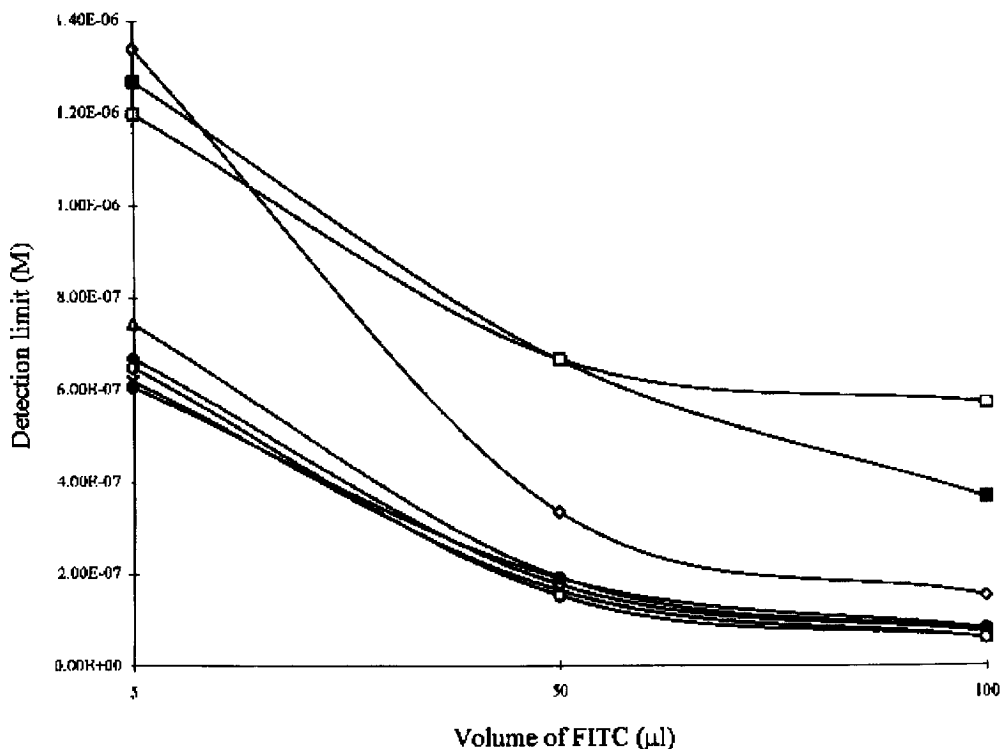


Fig. 1. Detection limits of biogenic amines obtained for different volumes of FITC solution ( $2.5 \cdot 10^{-3}$  M) to 1 ml of amine stock solution ( $10^{-4}$  M). Molar ratio of FITC to amine: 0.125 (5  $\mu\text{l}$ ); 1.25 (50  $\mu\text{l}$ ) and 2.5 (100  $\mu\text{l}$ ). Amine identities:  $\square$ =Spn;  $\blacksquare$ =Spd;  $\diamond$ =Tryp;  $\triangle$ =His;  $\blacklozenge$ =Put;  $\circ$ =Phe;  $\times$ =Tyr;  $\bullet$ =Cad.

unsatisfactory, Spd and Spn produced very broad peaks. Addition of 30 mM SDS improved greatly the resolution and peak shape. However, addition of 100 mM SDS resulted not only in longer migration times but loss in resolution. The optimum separation conditions for the FTC-amines were found to be 20 mM borate buffer pH 9.50, modified with 60 mM SDS. Fig. 2 shows an electropherogram of a mixture of polyamine standards obtained using the optimum conditions. The dissociation of the carboxylic group of FITC (pK 6.9) gives the FTC-amines a net negative charge under the conditions used. There-

fore, the partitioning process together with the electrophoretic retardation of the anions are essential factors affecting the optimum separation of the FTC-amines. As shown in Fig. 3, the SDS concentration significantly influences the migration order and resolution of the FTC-amines. Capacity factors ( $k'$ ) as a measure of partitioning of the solutes into the micellar phase were calculated accordingly to the equation [19]:

$$k' = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})}$$

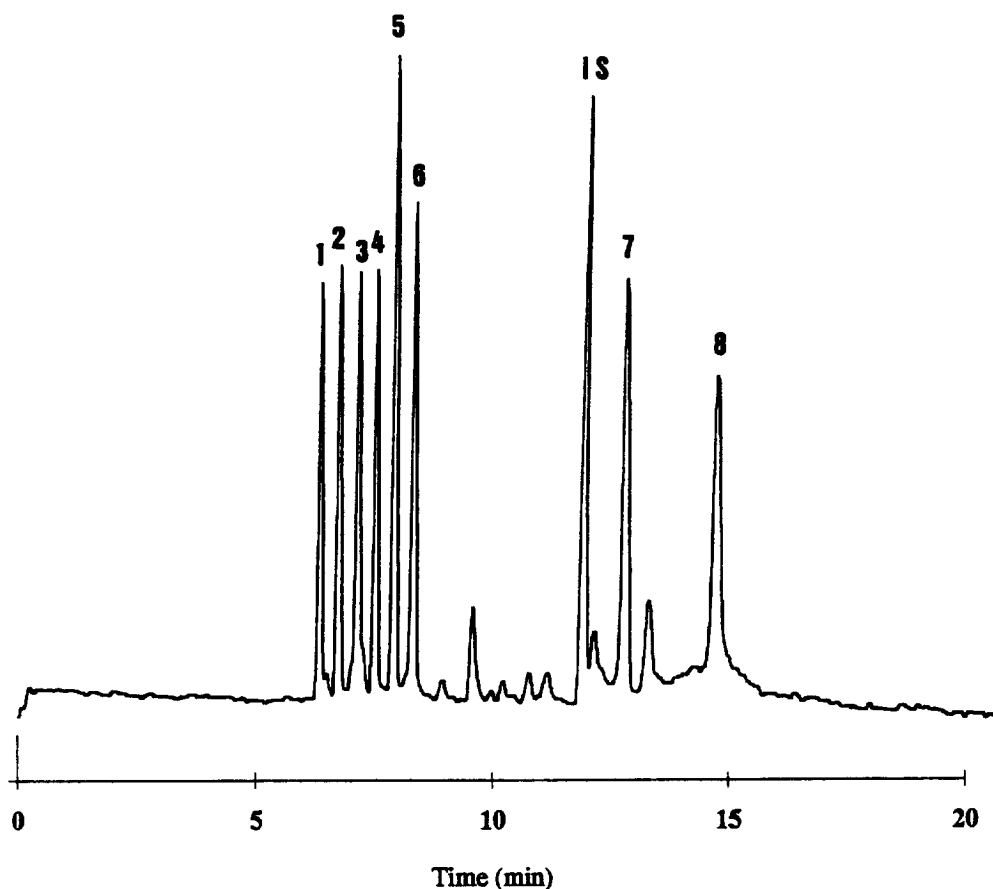


Fig. 2. Capillary electrophoretic separation of a mixture of FTC-amines. Conditions: buffer, 20 mmol/l borate pH 9.5, 60 mmol/l SDS; separation tube, 65 cm total length (effective length 50 cm)  $\times$  50  $\mu$ m I.D.; applied voltage, 22 kV; detection, excitation wavelength 488 nm, emission wavelength 519 nm; injection, hydrodynamic injection for 10 s; temperature, ambient. Peak identities: 1 = Put; 2 = His; 3 = Tyr; 4 = Cad; 5 = Phe; 6 = Tryp; 7 = Spd; 8 = Spn; I.S. = internal standard (1,7-diaminoheptane). Concentration of each peak is ca.  $10^{-5}$  mmol/l.

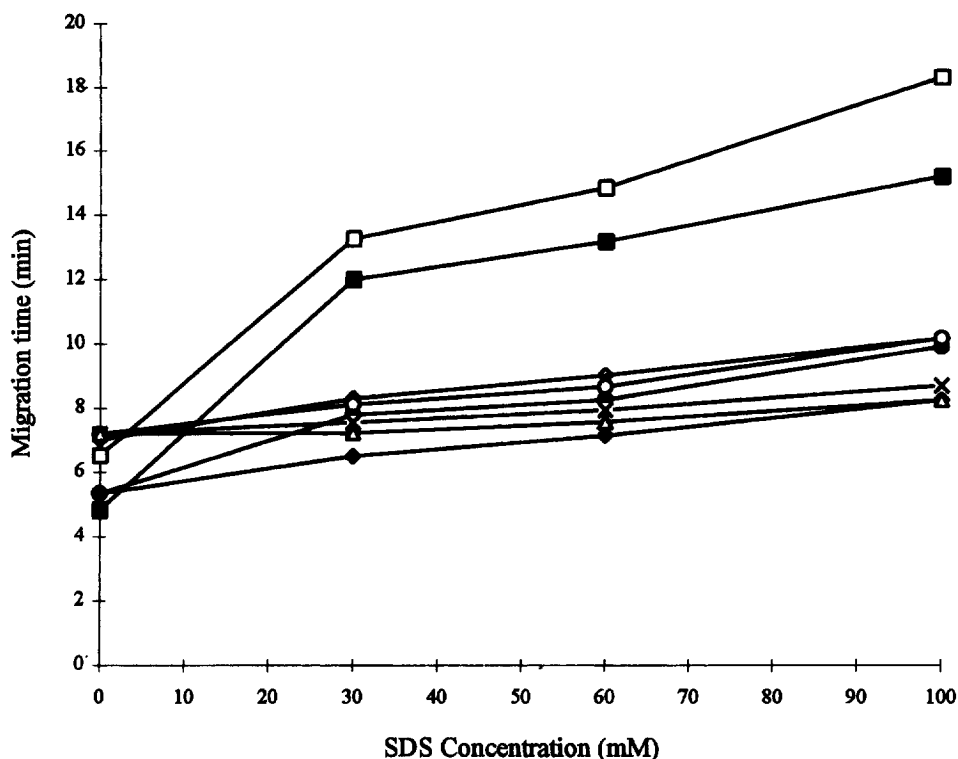


Fig. 3. Change in mobilities of the FTC-amines as function of SDS concentration. FTC-amine identities: □ = Spn; ■ = Spd; ◇ = Tryp; ○ = Phe; ● = Cad; △ = His; × = Tyr; ◆ = Put.

where  $t_0$  is the migration time in the absence of the micelles,  $t_R$  is the migration time of the analyte in the presence of the micelles, and  $t_{mc}$  is the migration time of the micellar phase. For the purpose of obtaining  $t_{mc}$ , we used Sudan III as marker. Since it was not possible to use the fluorescence detection system for Sudan III, the electrophoretic system was transferred to a UV detection system. Therefore, this value was considered to be an approximate value and it was not suitable for accurate determination of capacity factors. Nevertheless, capacity factors determined for the separation of the FTC-amines using the optimum concentration of SDS are listed in Table 1. The amines that show higher capacity factors are the linear ones which can react with several molecules of FITC. Spd and Spn exhibit very high capacity factors, probably due to the gain in hydrophobicity after derivatization with three and four

molecules of FITC, respectively. Selectivity for FTC-Spd and FTC-Spn was low in the absence of micelles. Therefore, solubilization and hydrophobic interaction with the micelles are probably the main mechanisms for the separation of these two amines. FTC-His exhibits a very low capacity factor although it carries two molecules of FITC. The reason may be that this amine has a free amino group and at the working pH is most likely positively charged. As a result it is less solubilized by the micelles. Furthermore, being less attracted towards the anode, FTC-

Table 1  
Capacity factors ( $k'$ )

Amine	His	Tyr	Phe	Tryp	Put	Cad	Spd	Spn
$k'$	0.19	0.31	0.60	0.73	0.74	1.22	9.52	18.20

His is expected to migrate faster. In the case of FTC-Tyr the presence of a hydroxylic group makes it more hydrophilic and therefore less easily solubilized by the micelles, thus explaining its low capacity factor.

### 3.3. Linearity and reproducibility

Precision was determined by performing repetitive analysis of the amine standard solutions ( $n=5$ ). Table 2 shows the relative standards deviations (% R.S.D.) in peak area and migration time. The values were below 5% for the peak area and below 0.50% for the migration times. These results indicate that the precision of the method is satisfactory. The linearity was determined from repetitive analysis ( $n=3$ ) at five different concentrations in the range of  $10^{-5}$  M to  $10^{-7}$  M (FITC ratio 2.5) using 1,7-diaminoheptane as internal standard. Calibration graphs were constructed and the regression equations of the plot and their correlation coefficients were determined as follow:

Putrescine:	$y = 116447x + 0.0941$	$r = 0.9941$
Histamine:	$y = 130870x + 0.0452$	$r = 0.9906$
Tyramine	$y = 160004x + 0.1072$	$r = 0.9941$
Cadaverine	$y = 160382x + 0.1006$	$r = 0.9928$
Phenethylamine	$y = 177255x + 0.1294$	$r = 0.9861$
Tryptamine	$y = 82063x + 0.0990$	$r = 0.9854$
Spermidine	$y = 105104x + 0.0871$	$r = 0.9927$
Spermine	$y = 182432x - 0.2874$	$r = 0.9882$

Table 2  
Relative standard deviation ( $n=5$ ) for the migration time (MT) and peak area (PA)

Amine	% R.S.D.	
	MT	PA
Put	0.17	1.75
His	0.50	2.03
Tyr	0.20	3.60
Cad	0.23	3.89
Phe	0.20	4.02
Tryp	0.25	2.75
Spd	0.31	5.74
Spn	0.47	2.81
I.S.	0.45	3.18

### 3.4. Application of the method to soy sauce samples

Two methods were tested for extraction: cationic exchange extraction using Amberlite GC-50 resin [9] and the liquid extraction method [16] described previously. Ion-exchange extraction gave a system peak that prevents the identification of several FTC-amines. Therefore the liquid extraction method was adopted. Biogenic amines present in soy sauce were analyzed and identified according to their electrophoretic mobility and increases in fluorescence intensity when the samples were spiked with FTC-amines at high concentrations. A typical electropherogram of FTC-amines in soy sauce is shown in Fig. 4. We identified three amines. These three amines plus tyramine have been identified before by other methods for another soy sauce sample [9]. However, we could not identify tyramine in the sample tested. In addition, it was found that the application of the method to real samples presents difficulties due to the interfering signals from N-containing compounds that reacted with FITC or from excess reagent or other impurities that migrate about the same time as the FTC-amine peaks. Due to this reason it was necessary to use the lowest concentration of FITC possible. On the other hand, the dependence of the fluorescence signal on the amount of FITC used in the derivatization reaction of the amines (see Fig. 1), and the presence of interfering compounds resulted in inaccuracies for the quantification of amines in real samples. Therefore, the application of this method to real samples will be investigated further after developing an effective procedure for removal of excess FITC and other interfering compounds.

## 4. Conclusion

In this study, the separation of eight biogenic amines by micellar electrokinetic chromatography with fluorescence detection and the applicability of the method to a real sample was investigated. Optimum separation was obtained using borate buffer modified with SDS. Capillary regeneration was effectively done by using an acid-oxidizing rinsing

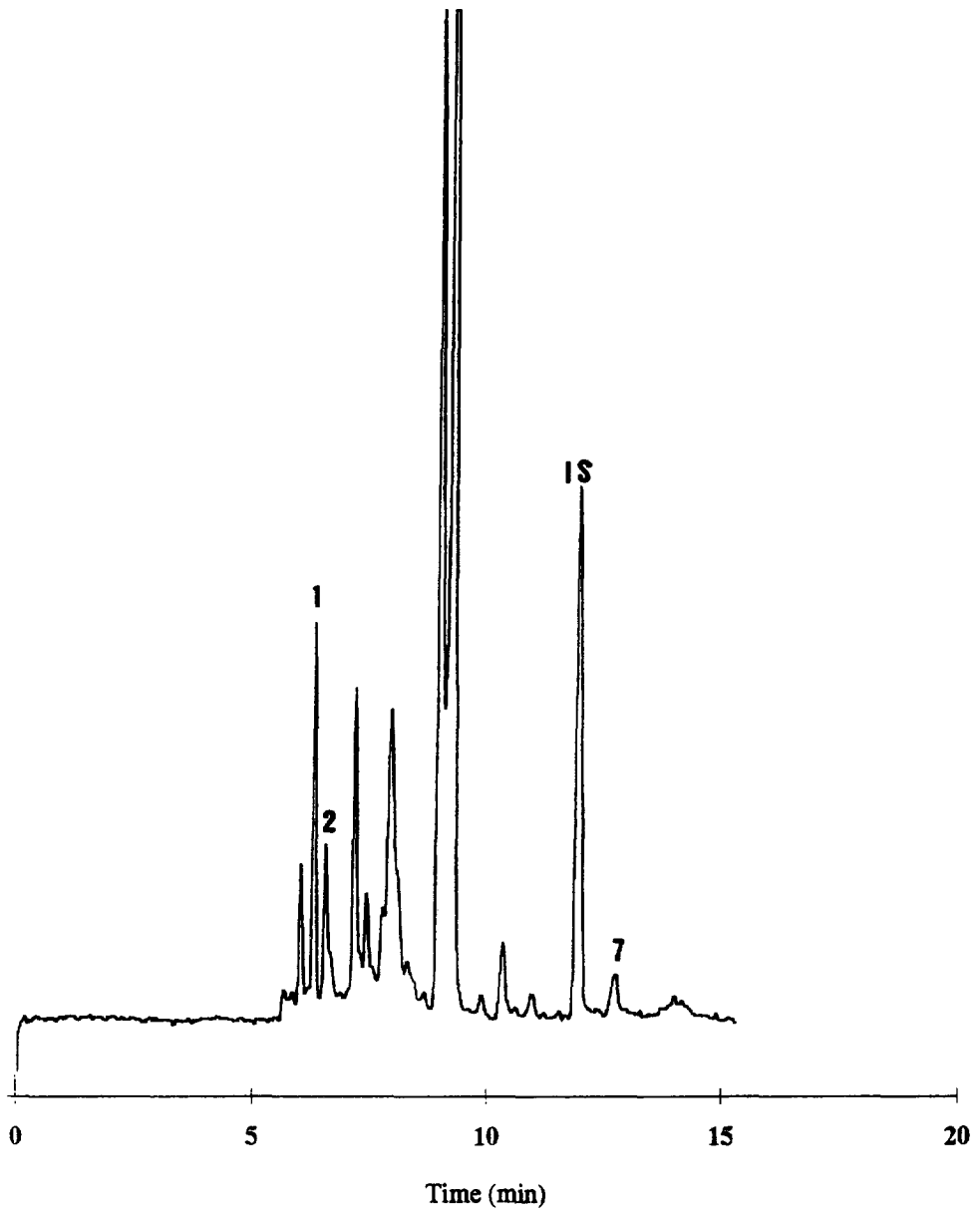


Fig. 4. Electrokinetic chromatogram obtained for the analysis of amines in a soy sauce sample. Peak identities: 1 = Put; 2 = His; 7 = Spd; I.S. = internal standard.

solution. Fluorescence detection was performed after derivatization of the amines with FITC. A detection limit of  $10^{-8}$  M was obtained for the FITC-amines.

The relative standard deviations for the migration time and the peak area were less than 0.5% and 5% respectively. Good linearity is observed from the

calibration plots at concentrations from  $10^{-5}$  M to  $10^{-7}$  M.

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