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Indirect fluorescence detection of aliphatic biogenic polyamines and diamines following chromatographic separation

Min Yang, Sterling A. Tomellini*

Department of Chemistry, University of New Hampshire, Durham, NH 03824, USA

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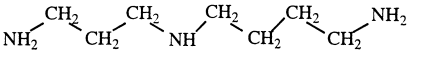
Abstract

A convenient, sensitive method has been developed for detecting aliphatic biogenic polyamines, 1,2-diamines and 1,3-diamines, following separation by HPLC. The method is based on a postcolumn, non-derivatization reaction using low cost reagents. The basis for this detection scheme is the fluorescence of L-tryptophan (L-Trp), which is quenched in copper(II)–L-Trp complexes; the fluorescence of L-Trp recovers with the addition of analytes that have a greater copper(II) affinity, such as aliphatic polyamines, 1,2-diamines and 1,3-diamines. Thus, the presence of these compounds in the HPLC eluent can be inferred by monitoring the fluorescence of L-Trp. pH has a strong effect on this detection system and this is discussed in detail. The detection limits following HPLC separation are 5 and 10 pmol injected for spermine and spermidine, respectively, with linear response regions to 1000 pmol.

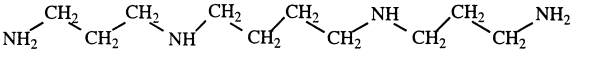
Keywords: Fluorescence detection; Detection, LC; Indirect detection; Polyamines; Biogenic amines; Spermine; Spermidine; Copper–tryptophan complexes; Amines

1. Introduction

Due to their biological importance, it is important to develop convenient and sensitive methods for the detection of the aliphatic biogenic polyamines and diamines. These compounds are naturally occurring compounds that are found in a wide variety of yeasts [1,2], plants [3,4] and animals [5–7]. Two biogenic polyamines, spermidine and spermine (Fig. 1), are important for rapid tissue growth. The concentrations of spermidine and spermine were shown to be related to several parameters of cell proliferation, such as the contents of proteins and nucleic acids in developing embryos [8]. Enhanced synthesis and accumulation of aliphatic polyamines in rapidly growing tissues occur prior to the synthesis of DNA [9]. Although the precise biological role of polyamines in this process is still under investigation, it has been suggested that these two polyamines, by virtue of their charged nature under physiological conditions and their conformational flexibility, might serve to stabilize macromolecules such as nucleic acids by



(a)



(b)

Fig. 1. Molecular structures for spermidine (a) and spermine (b).

*Corresponding author. Fax: +1-603-862-42-78.

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anion neutralization [10–12]. These aliphatic polyamines are also important in cancer research. It has been shown that polyamine patterns in tumor cells are significantly different from those of normal muscle tissues. For instance, the molar ratio of spermidine to spermine in Ehrlich ascites carcinoma was found to be strikingly lower than that of typical rapidly growing animal tissues [13]. Both the spermidine concentration as well as the molar ratio of spermidine to spermine are found to be elevated in RD/3 sarcoma compared with normal muscle tissues [14]. Attempts have also been made to use polyamines for the detection of malignancy [15]. Diamines are important in the synthesis and degradation pathways for amino acids in biological systems. Furthermore, elevated concentrations of certain diamines in blood can be indicative of specific carcinomas and, in organ transplant recipients, evidence of physiological rejection of transplanted tissues [8,16]. Diamines are also widely recognized as indicators of spoilage in foods, especially in seafood and other meats [17,18].

Detection of aliphatic amines following HPLC separation has been widely studied. Most natural aliphatic biogenic polyamines do not possess naturally occurring chromophoric or fluorophoric moieties, which is why they are usually derivatized prior to chromatographic separation, to facilitate photometric detection. Detection methods that require derivatization have several disadvantages, including the need for additional sample processing, variability in reaction completeness, decomposition of the derivatization products, and toxicity of some derivatizing agents. For these reasons, direct detection without derivatization is preferred whenever possible.

A variety of ways have been developed to detect the aliphatic amines without derivatization. Chemiluminescence detection of aliphatic amines following chromatographic separation has been achieved using the reaction of tris(2,2′-bipyridyl)ruthenium(II) with the analytes [19], based on a chemiluminescent oxidation–reduction reaction mechanism. Indirect detection of various biogenic aliphatic polyamines has been achieved in ion-exchange chromatography [20] and capillary electrophoresis [21] without the need for derivatization. By exploiting the property that the amines exist as ions at appropriate pH values, UV-absorbing ionic species were added to the mobile phase, not only to elute the analytes but also to monitor the presence of the analytes. As the ionic amines elute from the column, the concentration of the counter ions in the mobile phase, which are being monitored, decreases to maintain electroneutrality in the mobile phase. However, one problem with this approach is that it suffers from high detection limits, which limits its application for real biological samples. Recently, integrated pulsed amperometric detection (IPAD), a newly developed variant of the pulsed amperometric detection method, has been used to detect underivatized biogenic monoamines and diamines following HPLC separation [22–24]. In this detection mode, current is integrated continuously during a cycle in which the electrode is oxidized and then reduced to the original state. The advantage of IPAD is that, by canceling the charge from oxide formation and reduction, the effect on the baseline is greatly minimized.

The detection approach presented here is based on monitoring the fluorescence of the amino acid, L-tryptophan (L-Trp). It has been shown that the fluorescence of L-Trp is quenched in the presence of copper(II) [25,26]. Quenching is due to the formation of a L-Trp–copper complex. With the addition of aliphatic polyamines and diamines, which have a greater affinity for copper(II) than L-Trp, L-Trp is freed from the complex, resulting in the recovery of L-Trp fluorescence, as shown by:

$$\text{Cu(L-Trp)}_2 + n\text{X} = \text{Cu(X)}_n + 2\ \text{L-Trp} \quad (1)$$

where X is the analyte molecule and n is the number of analyte molecules coordinating to Cu(II). Thus, the presence of these analytes can be inferred by monitoring the fluorescence of L-Trp, as described by the following equations:

$$[\text{X}] = 2/n \times [\text{L-Trp}] \quad (2)$$

$$[\text{X}] = K \times 2/n \times FLUO \quad (3)$$

where [X] and [L-Trp] are concentrations of the analytes and L-Trp, respectively, K is an arbitrary constant and FLUO is the fluorescence intensity of L-Trp.

We present here a convenient and sensitive implementation of a postcolumn detection scheme for monitoring spermidine and spermine, as well as 1,2-diamines and 1,3-diamines, using Cu(L-Trp)_2 as the

postcolumn reagent. A discussion of the reaction conditions and the detection mechanism will be presented in detail.

2. Experimental

2.1. Reagents

The biogenic polyamines, spermine [manufacturer's stated purity, 99.2% by thin-layer chromatography (TLC)] and spermidine (manufacturer's stated purity, 99.2% by GC), and L-Trp (manufacturer's stated purity, 99% by TLC) were purchased from Sigma (St. Louis, MO, USA). The 1,2-diamines, ethylenediamine, *N*-methylethylenediamine and the l,3-diamine, 1,3-aminopropane (all with a manufacturer's stated purity of 99.0% by GC) were also purchased from Sigma. Reagent-grade copper sulfate, sodium acetate and sodium borate were purchased from Baker (Phillipsburg, NJ, USA).

The deionized water used in the preparation of standard solutions and eluents was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile phases were filtered through a 0.45-μm nylon filter (Whatman, Hillsboro, OR, USA) prior to use. Dilute aqueous solutions of reagent-grade sodium hydroxide (Fisher, Pittsburgh, PA, USA) and hydrochloric acid (Baker) were used to adjust the pH of the mobile phase and postcolumn reagent.

2.2. Apparatus

A Perkin-Elmer Model 204 fluorescence spectrophotometer (Norwalk, CT, USA) was used for the initial fluorescence studies. The excitation wavelength and analyzer wavelength were set at 280 and 350 nm, respectively. The chromatographic separations were performed using a Nicolet LC9560 HPLC system (Madison, WI, USA) equipped with a Rheodyne Model 7125 injector (Cotati, CA, USA) fitted with a 10-μl injection loop. Separation of the polyamines was achieved with the use of a Hamilton PRP-X200 column (250×4.6 mm I.D.; Reno, NV, USA) containing a polymer-based strong cation-exchanger. A Hitachi 655A-11 LC pump (Tokyo, Japan) delivered the postcolumn reagent via a mixing tee. A Varian flow control damper (P/N 03-905320-00; Palo Alto, CA, USA) was placed between the postcolumn reagent pump and the mixing tee to improve flow stability. A Kratos Spectroflow 980 fluorescence detector (Ramsey, NJ, USA) fitted with a 10-μl detection flow cell was used for chromatographic detection. The excitation and analyzer wavelength of the detector were set at 280 and 350 nm, respectively.

3. Results and discussion

3.1. pH optimization

The pH of the solution in the detector flow cell is a very important parameter that needs to be controlled for several reasons. The fluorescence of Cu(L-Trp)$_2$ is pH-dependent since the extent of Cu(II) and L-Trp complexation is a function of pH, and the degree of fluorescence quenching is directly related to the extent of complexation. The complexing affinities of the polyamines to Cu(II) are also dependent on the solution's pH, with only deprotonated forms of polyamines coordinating to Cu(II). Based on these considerations, the pH needs to be adjusted so that the background fluorescence of the solution is relatively low, thus providing a 'dark' background, while at the same time providing high complexing affinities for the analytes. It should be noted that the optimum pH for fluorescence quenching is not necessarily the pH that results in the optimum complexing affinities of the polyamines. Also, the optimum detection pH for one particular polyamine might not be the optimum detection pH for other polyamines.

The fluorescence profile of a 0.5×10^{-4} *M* solution of Cu(L-Trp)$_2$ as a function of solution pH ranging from 4 to 11 is shown in Fig. 2. As can be seen from the plot, the fluorescence reaches a minimum value at pH 8.1, at which point approximately 95% of the fluorescence of the L-Trp is quenched.

The equations governing the equilibria that exist in solutions of Cu(II) and L-Trp are given below:

$$Cu^{2+} + A^{-} \rightleftharpoons [CuA]^{+} \quad (4)$$

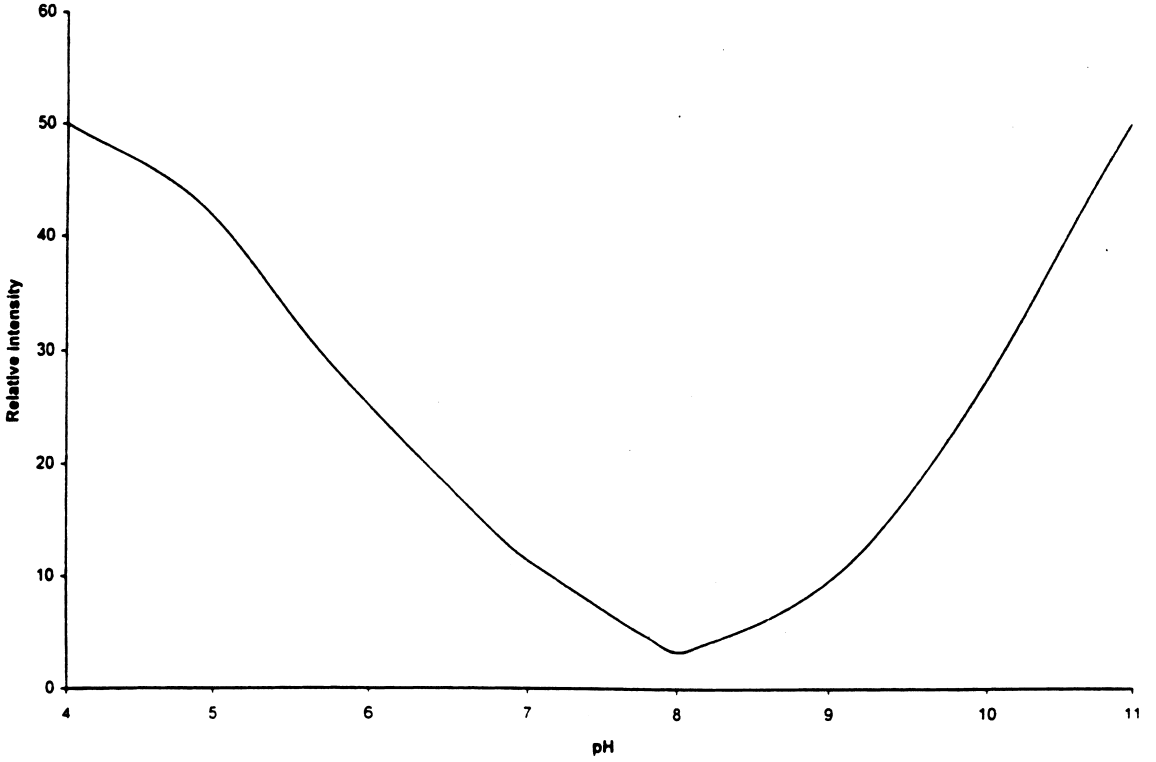

Fig. 2. Fluorescence of a 0.5×10^{-4} *M* Cu(L-Trp)$_2$ solution as a function of pH.

$$Cu^{2+} + 2A^- \rightleftharpoons CuA_2 \quad (5)$$

$$A^- + H^+ \rightleftharpoons HA \quad (6)$$

$$HA + H^+ \rightleftharpoons H_2A^+ \quad (7)$$

$$Cu^{2+} + OH^- \rightleftharpoons CuOH^+ \quad (8)$$

$$Cu^{2+} + 2OH^- \rightleftharpoons Cu(OH)_2 \quad (9)$$

$$Cu^{2+} + 3OH^- \rightleftharpoons Cu(OH)_3^- \quad (10)$$

$$Cu^{2+} + 4OH^- \rightleftharpoons Cu(OH)_4^{2-} \quad (11)$$

$$2Cu^{2+} + 2OH^- \rightleftharpoons Cu_2(OH)_2^{2+} \quad (12)$$

A = L-Trp

For a given Cu(L-Trp)$_2$ system, in addition to the complexation equilibria between Cu(II) and L-Trp (Eqs. (4) and (5)), there are also protonation equilibria (Eqs. (6) and (7)) of L-Trp and complexation equilibria (Eqs. (8)–(12)) between Cu^{2+} and OH^-, which compete for Cu^{2+} and L-Trp. The complexation between Cu(II) and L-Trp is optimal at pH 8.1, at which point, the sum of the effects of the competitions from the protonation of L-Trp, and the complexation between Cu(II) and OH^-, is minimal, resulting in optimum fluorescence quenching. The increase in fluorescence of the solution below pH 8.1 is due to the increasing protonation of L-Trp, while increased fluorescence above pH 8.1 is the result of increasing complexation between Cu(II) and OH^-. Although pH 8.1 may not be the ideal pH for detection, it is anticipated that the optimum will be close to this value, otherwise, the dissociation of the Cu(L-Trp)$_2$ will be so substantial that the introduced analyte molecules will tend to react with other forms of Cu(II) instead of Cu(L-Trp)$_2$. The dissociation can also result in a higher fluorescence background level and increased noise.

For an optimized chromatographic separation

scheme, the pH of the solution in the detector cell was adjusted by changing the pH of the postcolumn reagent. To experimentally determine the optimal detection pH for spermine and spermidine, mixtures of the two were injected into HPLC, separated, and the fluorescence signal intensities obtained were plotted as the function of the pH values of the detected solutions (Fig. 3). The figure shows that the fluorescence signals of the two polyamines changed significantly in the pH range of 7.4 to 9.2. For spermidine, the signal increases steadily in this pH range; for spermine, the fluorescence signal increases to a maximum value at pH 8.65 and then slowly decreases. These pH-dependent patterns can be explained with the following considerations. There are three factors affecting the signal intensities of these two aliphatic polyamines: (1) the dissociation of Cu(L-Trp)$_2$ increases both above pH 8.1 and below pH 8.1; (2) the fluorescence of free L-Trp steadily increases above pH 8.3, due to the fact that the deprotonated form of L-Trp has a higher fluorescence efficiency than zwitterionic L-Trp [27] and (3) the affinities of the polyamines for Cu(II) vary as the pH changes, since complexation is affected by both the degree of polyamine protonation and Cu(II)–hydroxide complexation. To determine how the pH affects Cu(II)–polyamine complexation, the conditional stability constants, K_f', of the two complexes, Cu(spermine) and Cu(spermidine), were calculated using the following formula at different pH values:

$$K_f' = K_f \, \partial_A \, \partial_{cu^{2+}} \quad (13)$$

in which K_f' and K_f are the conditional stability constant and the stability constant of the complex, respectively; ∂_A is the fraction of unprotonated polyamines, and $\partial_{cu^{2+}}$ is the fraction of free Cu^{2+}

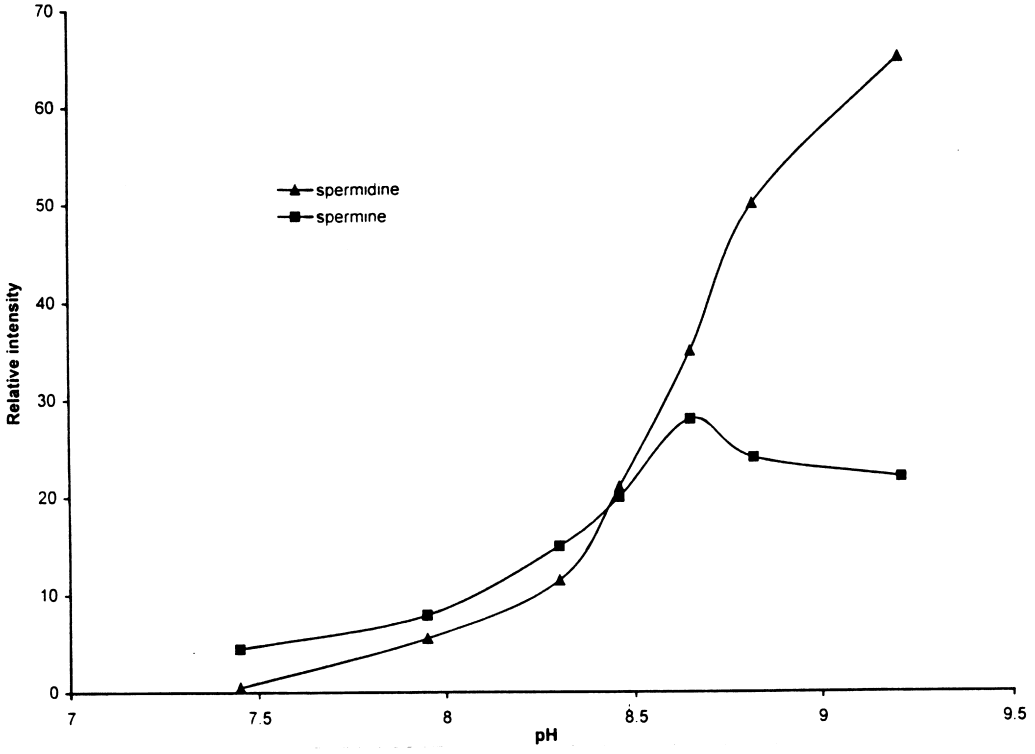

Fig. 3. Signal intensities of spermine and spermidine following chromatographic separation as a function of detection pH. Separation was achieved on a Hamilton PRP-X200 SCX column at ambient temperature. The mobile phase was 0.8 *M* KCI, buffered at pH 5.25 with 1.5×10^{-3} *M* NaAc. The postcolumn reagent was 0.5×10^{-4} *M* Cu(L-Trp)$_2$ in a 4×10^{-3}-*M* sodium borate buffer. The eluent and postcolumn reagent flow-rates were 0.8 and 2.0 ml/min, respectively. The amounts of sample injected were 320 pmol for spermidine and 160 pmol for spermine.

with the consideration of Cu(II)–hydroxide complexation. The published p*K*a values of the two polyamines were obtained from Ref. [28]. The published K_f values of Cu(spermine) and Cu(spermidine) were obtained from Refs. [29,30] respectively. From the K_f' vs. pH plots presented in Fig. 4, it can be seen that, of the two complexes, Cu(spermine) has a maximum K_f' at pH 9.75 and Cu(spermidine) has a maximum K_f' at pH 9.30. Clearly, the complexing affinities of both of the polyamines tend to increase in the pH range 7.4 to 9.2.

Of the three factors affecting signal strength cited above, the first one tends to lead to a decrease in fluorescence signal intensity when the detection solution pH is away from 8.1, while the last two factors indicate that increasing pH enhances the signal intensity. The pH dependence patterns of the signals in Fig. 3 are the combined results of those three factors. Based on the data obtained, it can be concluded for spermine that the first factor dominates at pH values higher than 8.6, thus resulting in a decrease in signal intensity; while for spermidine, the combined effect of factors two and three dominates in the pH range from 7.4 to 9.2.

3.2. Stoichiometric study of the detection reaction

Solutions containing different concentrations of the aliphatic polyamines, spermine or spermidine, and three diamines, ethylenediamine, *N*-methylethylenediamine or 1,3-aminopropane were added to standard 0.5×10^{-4} *M* Cu(L-Trp)$_2$ solutions containing 4 m*M* sodium borate adjusted at pH 8.1. This resulted in the 'titration' of the Cu(L-Trp)$_2$ reagent with these compounds. Representative plots of fluorescence intensity versus the concentration of spermine and ethylenediamine are given in Fig. 5. Both of these plots show that the recovered fluorescence signal reaches a plateau when a stoichiometric amount of compound is added to the detection reagent. A stoichiometric ratio of 1:1 for [spermine]–[Cu(L-Trp)$_2$] is determined from the titration data, within experimental error. This corresponds to the reaction:

$$\text{Cu(L-Trp)}_2 + \text{spermine} = \text{Cu(spermine)} + 2\ \text{Trp} \quad (14)$$

Similar titration data obtained for spermidine also indicate a stoichiometric ratio of 1:1 for [spermidine]–[Cu(L-Trp)$_2$].

However, a stoichiometric ratio of 2:1 for [ethylenediamine]–[Cu(L-Trp)$_2$] was determined from the titration data of ethylenediamine, suggesting that the reaction occurs

$$\text{Cu(L-Trp)}_2 + 2\ \text{ethylenediamine} = \text{Cu(ethylenediamine)}_2 + 2\ \text{Trp} \quad (15)$$

The same stoichiometric ratio is indicated for both *N*-methylethylenediamine and 1,3-aminopropane. The discrepancy between the two stoichiometric ratios can be explained by the fact that the two biogenic polyamines have more amino groups than the diamines, resulting in a larger complexing capacity.

Thus, every spermine or spermidine molecule produces two fluorescent L-Trp molecules, while every 1,2-diamine or 1,3-diamine molecule displaces only one fluorescent L-Trp molecule.

3.3. Chromatographic detection

Based on these results, a system for detecting spermidine and spermine, 1,2-diamines and 1,3-diamines after separation by HPLC was developed. A Cu(L-Trp)$_2$-based fluorescence detection scheme for these compounds was implemented following the chromatographic separation. Separation of spermine and spermidine was achieved isocratically within 5 min using a mobile phase containing 0.8 *M* potassium chloride and 1.5×10^{-3} *M* acetate buffer adjusted to pH 5.25. A solution containing 5×10^{-3} *M* Cu(L-Trp)$_2$ in 4×10^{-3} *M* sodium borate buffered at pH 8.50 was added postcolumn. Separation of the diamines was achieved at lower potassium chloride concentrations. The concentration of sodium borate, the postcolumn reaction buffer, was set higher than that of the acetic acid, the mobile phase buffer, to assure a larger buffering capacity for the former. Caution should be taken in the selection of the postcolumn buffer, since some buffer candidates in the pH range from 8 to 10 are amines, such as the glycine–NaOH buffering system, which could potentially interfere with analyte detection by competing with polyamine analytes for Cu(II). A representative

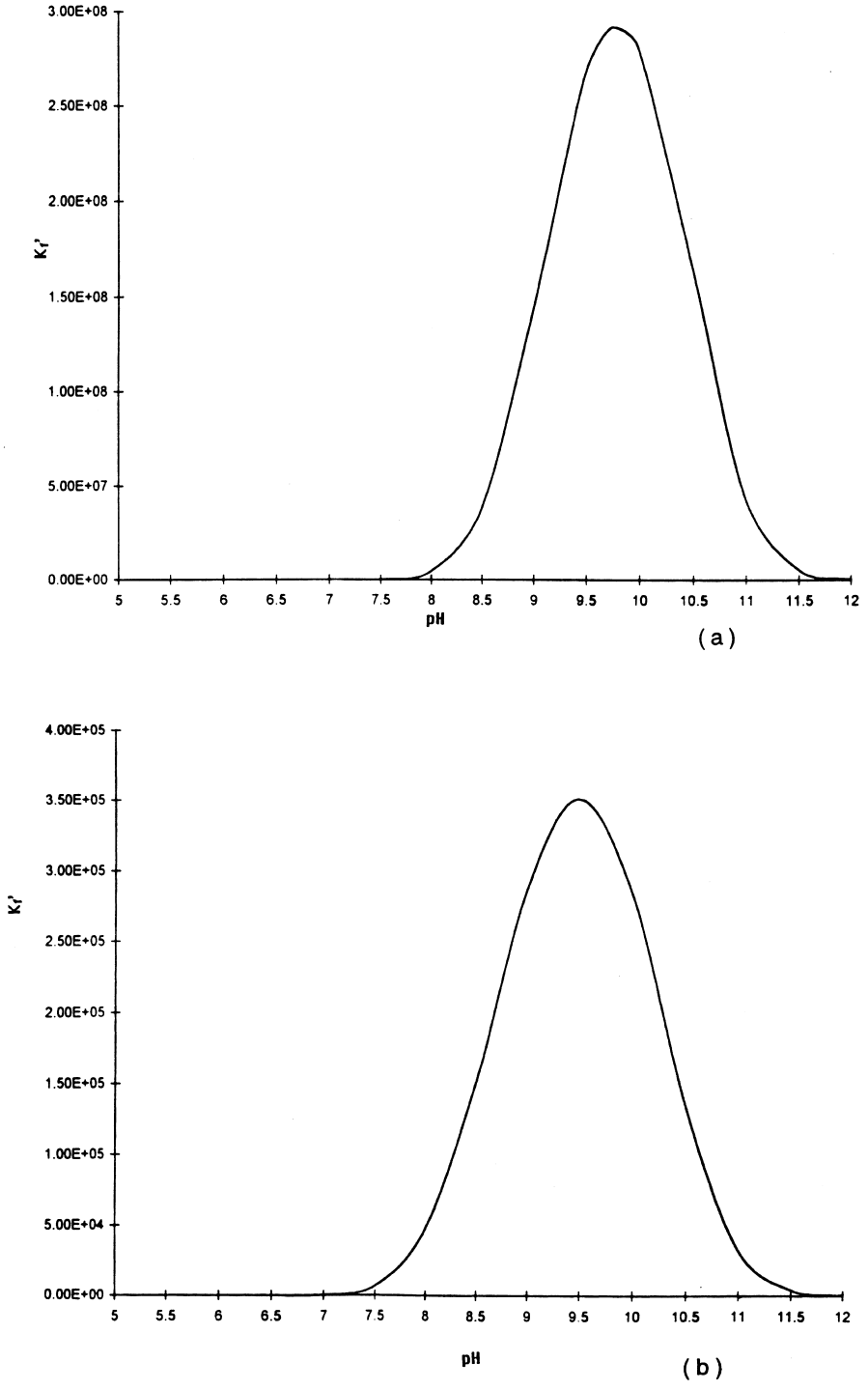

Fig. 4. Calculated conditional stability constants (K_f') of (a) Cu(spermine) and (b) Cu(spermidine) as a function of pH.

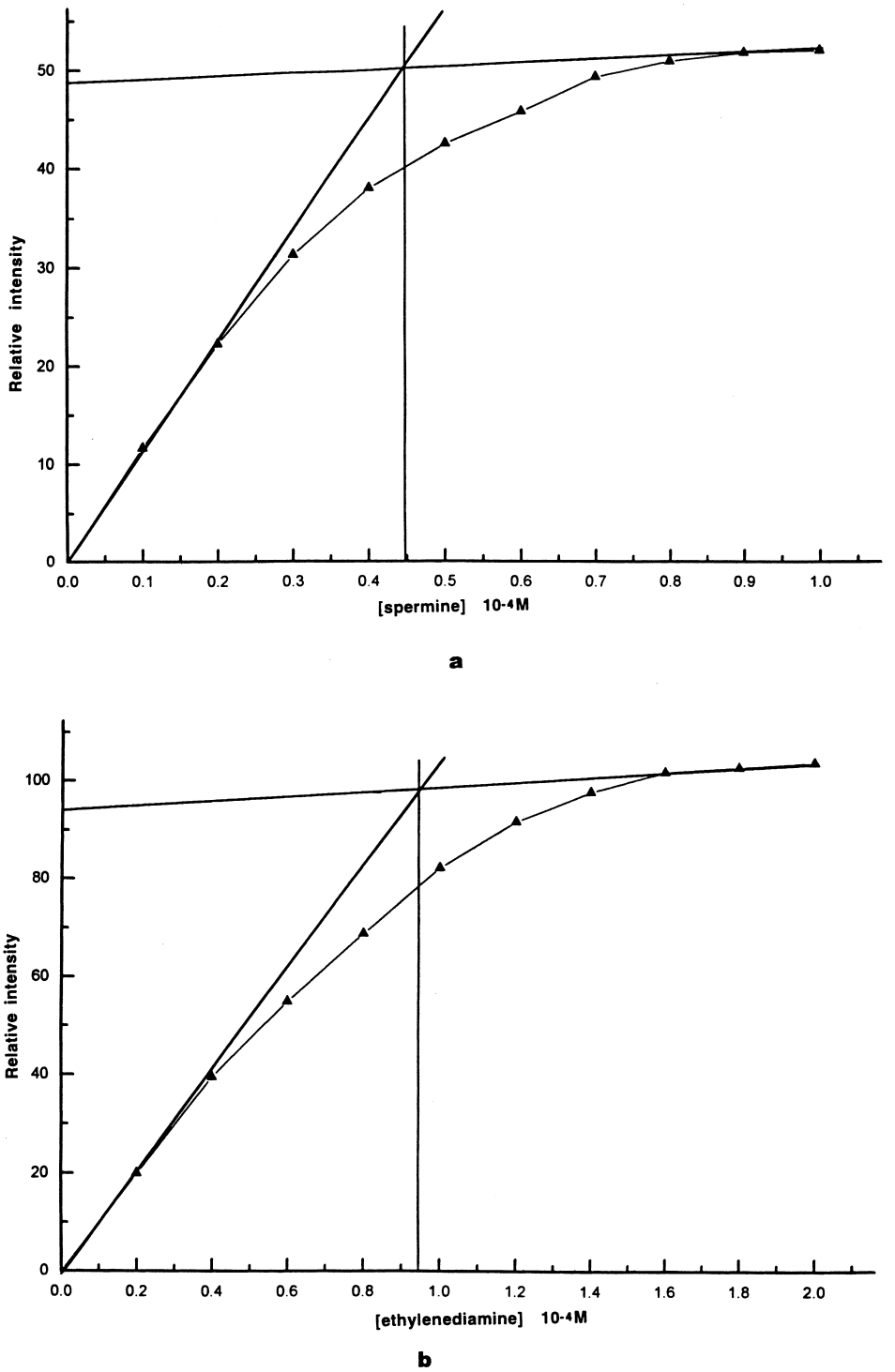

Fig. 5. Stoichiometric study of the reaction of Cu(L-Trp)$_2$ with (a) spermine and (b) ethylenediamine. The concentration of Cu(L-Trp)$_2$ is 0.5×10^{-4} *M*, adjusted to pH 8.1, in 4×10^{-3} *M* sodium borate.

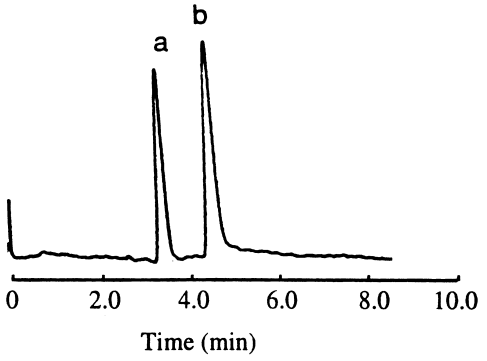

Fig. 6. Representative chromatogram of spermine and spermidine. Separation was conducted using a Hamilton PRP-X200 SCX column at ambient temperature. The mobile phase was 0.8 *M* KCl adjusted to pH 5.25 with 1.5×10^{-3} *M* acetate buffer. The postcolumn reagent was 0.5×10^{-4} *M* Cu(L-Trp)$_2$, buffered at 8.50 with 4×10^{-3} *M* sodium borate. The eluent and postcolumn reagent flow-rates were 0.8 and 2.0 ml/min respectively. Peak a, spermidine, 640 pmol; peak b, spermine, 320 pmol.

chromatogram of spermine and spermidine, obtained under optimized detection conditions, is presented in Fig. 6. Representative chromatograms of the diamines are presented in Fig. 7.

The fitted parameters for the working curves obtained for spermine and spermidine, the detection limits ($S/N=3$) and associated precision, expressed as the relative standard deviation (RSD), which was obtained by analyzing samples containing concentrations within the range of the working curve for each polyamine, are given in Table 1. The results indicate that the detection limits for these two polyamines, using the developed method, compare favorably with those using other reported derivatization methods [31,32]

4. Conclusions

A sensitive and convenient way of detecting the biogenic aliphatic polyamines, 1,2-diamines and 1,3-diamines following chromatographic separation has been developed. The method may be applicable to other amines, although a limiting factor is the stability constant for forming the Cu(II) complex. For example, the compound putrescine, which has two amino groups separated by four methylene groups, was found to be much less detectable than either spermine or spermidine under the same experimental conditions, possibly due to the fact that substantial structural distortion is required for it to coordinate with Cu(II).

It is anticipated that further improvements in analyte detection can be achieved by employing other fluorophores. The Cu(L-Trp)$_2$ complex does fluoresce to a limited extent, even in the presence of excess Cu(II), resulting in an increase in background noise. Furthermore, L-Trp is not a highly fluorescent compound, which also increases the detection limits of the analytes, since detection is proportional to the magnitude of the fluorescence signal.

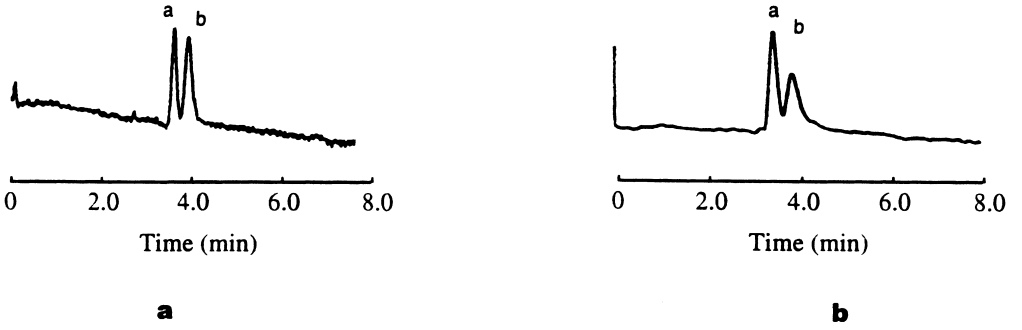

Fig. 7. (a) Chromatograms of ethylenediamine (a) and l,3-aminopropane (b), 300 pmol each. The mobile phase was 0.4 *M* KCl adjusted to pH 5.25 with 1.5×10^{-3} *M* acetate buffer. (b) Chromatograms of ethylenediamine (a) and *N*-methylethylenediamine (b), 700 pmol each. The mobile phase was 0.5 *M* KCl adjusted to pH 5.25 with 1.5×10^{-3} *M* acetate buffer. Separation was conducted using a Hamilton PRP-X200 SCX column at ambient temperature. The postcolumn reagent was 0.5×10^{-4} *M* Cu(L-Trp)$_2$, buffered at 8.50 with 4×10^{-3} *M* sodium borate. The eluent and postcolumn reagent flow-rates were 0.8 and 2.0 ml/min. respectively.

Table 1
Characteristic parameters of the calibration graphs and analytical figures of merit in the determination of polyamines

Polyamine	Linear range (pmol)	Regression equation[a]	r[b]	DL[c] (pmol)	RSD[d] (%)
Spermine	5–1000	$H=0.608+0.193\times C$	0.9991	5	4.6
Spermidine	10–1000	$H=0.745+0.074\times C$	0.9964	10	3.5

[a] H, peak height; C, analyte in pmole).
[b] Correlation coefficient.
[c] Detection limit.
[d] Relative standard deviation.

It is also likely that this detection method can be extended to other classes of analytes, including the amino acids. Most natural amino acids have good Cu(II) affinity under appropriate experimental conditions [33]. Preliminary results indicate that detection of these compounds can be achieved with detection limits comparable to those for spermine and spermidine, following chromatographic separation using this indirect fluorescence method.

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