# Separation of Biojenic Amines Using Dansyl Cloride Derivatization and Mixed Micellar Liquid Chromatography

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**ABSTRACT:** *Separation of some biogenic amines via RP-HPLC using mixed micellar mobile phase was investigated. The compounds were derivatized before hand by dansyl chloride as a chromophoric reagent. Appropriate conditions for separation, were determined by studying factors such as temperature, type and percentage of organic modifier, concentration of surfactants (SDS and Brij-35) and the pH of the mobile phase. The number of theoretical plates (N), asymmetry factor (B/A) and selectivity factor (α) considered as criteria for finding the appropriate conditions for separation. Appropriate condition, were: 40˚C, 40 mM SDS, 0.5 mM Brij-35 and 10% 1-propanol at pH=5.*

**KEY WORDS:** *Biogenic amines, HPLC, Micellar liquid chromatography, Mixed micelles.*

## INTRODUCTION

Biogenic amines are compounds formed by amino acid decarboxylation in fermented food. These compounds that occur in plants and in fermented foods, are produced by a number of microorganisms, and play important physiological roles in mammals. High amounts of some exogenous amines in the human diet may contribute to a wide variety of toxic effects. Reversed-phase HPLC is usually considered as the most suitable technique for analysis of amines [1-5]. Micellar liquid chromatography (MLC) is a mode of RP-HPLC, which uses aqueous solutions of surfactants at concentrations above their critical micelle concentration (CMC) as the mobile phase [6]. The micellar mobile phase eliminates the use of high concentrations of organic solvents currently used in separations by RP-LC. Micelles have been accepted as a microscopic medium, which provides a new basis for development of separation techniques. Micelles in the mobile phase are regarded as being a pseudo phase into which water-insoluble organic solutes are distributed, thus providing an effective means for controlling the elution of analytes [7]. Solute retention in MLC is determined by three competitive equilibriums: the micelle's pseudo phase, the bulk aqueous solvent and the stationary phase [6, 8]. Some advantages of the MLC technique as compared to conventional RP-HPLC include the low cost, non-flammability, non-toxicity and easy disposal of the mobile phase. Addition of an organic solvent, such as a short-chain alcohol, to the micellar

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mobile phase, increases separation efficiency [9]. Also, the pH of the micellar mobile phase affects retention and selectivity in MLC [10]. The use of mixed micellar solution in separation of organic compounds are also reported [11-13].

In this work, appropriate conditions were sought for separation of biogenic amines. These include: temperature, type and concentration of organic modifier, surfactants concentration (SDS and Bridj-35) and the pH of mobile phase. Since biogenic amines do not absorb UV-Vis, they were first derivatized by dansyl chloride as an absorbent chromophore.

## EXPERIMENTAL

### *Chemicals*

Dansyl chloride, trichloroacetic acid and ammonium acetate were purchased from Merck (Darmstadt, Germany). Other materials such as amines (methylamine, ethylamine, diethylamine, isopropyl amine, morpholine, putresine and buthylamine), surfactants (SDS and Brij-35), 1-propanol were obtained from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade. Mobile phase solutions were made in doubly distilled deionized water and filtered through 0.45 μm Millipore solvent filter.

### *Methods*

All stock standard solutions of amines (0.01 M) were prepared in 0.02 M HCl. Dansyl chloride (DNS-Cl) solution was made by dissolving 1g of DNS-Cl in 100 ml acetone. To derivatize amines with DNS-Cl, 2 ml of each amine solution was pipetted into a 25-ml flask. Subsequently, 2 ml of 5% trichloroacetic acid, 2 ml of 5% $Na_2CO_3$ and 2 ml of DNS-Cl solution were added and mixed in the reaction vessel. The reaction vessel closed and mixed for 90 min at 50˚C in water bath. At last, derivatized amines diluted by methanol and injected to HPLC.

### *Equipment*

The chromatographic measurements were carried-out on an HPLC system equipped with LC pump series 10, model 7125 manual injector with a 10μl loop and LC-95 UV-Vis spectrophotometer as detector (all from Perkin-Elemer, Norwalk, CT, USA). The analytical column used was $C_{18}$ (250 × 4.6mm, 10μm Waters, Milford, Mt, USA). pH meter used for pH adjustment of the mobile phases was 3030 (Jen Way, Ltd, UK). The column temperature was controlled by a water circulator bath. The Eluted dansylated amines were monitored at wavelength 254 nm.

### *Definition of chromatographic parameters*

Chromatographic parameters used in this paper were capacity factor (K') , selectivity factor (α), asymmetry factor (B/A) and the number of theoretical plates (N). Definition and determination methods for these parameters are as the following:

$K' = ( t_R - t_o ) / t_o$

Where $t_R$ and $t_o$ are retention times for the retained and nonretained compounds.

$\alpha = K'_2 / K'_1$

Where $K'_1$ and $K'_2$ are capacity factors for the neighboring compounds 1 and 2 ($k'_2 > k'_1$).

To determine asymmetry factor (B/A) for a chromatographic peak, a perpendicular line was drawn from the peak vertex to x axis ( or time axis ). This divides the peak width in two parts. The ratio of width part on the right hand side to the left hand side is considered as B / A.

The number of theretical plates (N), was calculated from the following equations:

$N = 5.54 ( t_R / W_{0.5} )^2$ ( for symmetric peaks )

and

$N = 41.7 (t_R / W_{0.1})^2 / (B/A + 1.25)$ (for asymmetric peaks)

where $t_R$ and $W_{0.5}$ and $W_{0.1}$ are retention time for the compound peak width at 50 percent and 10 percent from base line respectively.

## RESULTS AND DISCUSSION

### *The effect of temperature on efficiency*

The temperature of the column affects the pressure, analysis time and separation. The lower efficiency of MLC as compared with RP-LC is due to higher viscosity of micellar mobile phase, thus efficiency could be improved by increasing the temperature. This effect is due to faster mass transfer of solute between mobile and stationary phases [14]. The appropriate temperature was determined using the number of theoretical plates (N) and asymmetry factor (B/A) for dansylated isopropyl amine and methylamine. Table 1 shows that the maximum N and minimum B/A are obtained at 40˚C.

***Table 1: The effect of temperature on chromatographic parameters; N, B/A and K´.***

| | dansylated methylamine | | | dansylated iso-propylamine | | |
|---|---|---|---|---|---|---|
| Temperature (°C) | K´ | B/A | N | K´ | B/A | N |
| 25 | 5.4 | 1.9 | 423 | 7.5 | 1.6 | 710 |
| 30 | 5.3 | 1.9 | 450 | 7.4 | 1.6 | 714 |
| 35 | 5.0 | 1.8 | 510 | 7.2 | 1.5 | 722 |
| 40 | 4.8 | 1.7 | 556 | 6.9 | 1.4 | 732 |

*Conditions: mobile phase; 50 mM SDS + 1 mM Brij-35 + 5% 1-prOH; pH=7; flow rate 1.5 ml/min; column C18 (250 × 4.6 mm, 10µm).*

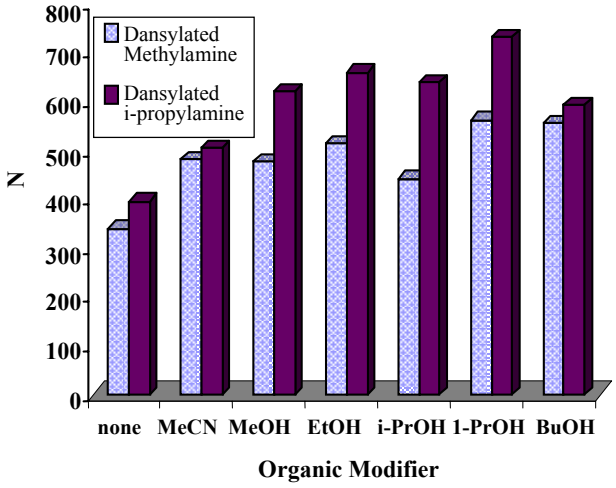



***Fig. 1: The effect of organic modifiers type on number of theoretical plate. Conditions: mobile phase; 50 mM SDS + 1 mM Brij-35, 5%(v/v) organic modifier, column; $C_{18}$ (250×4.6 mm) 10µm, pH=7, T =40˚C, flow rate 1.5 ml/min.***

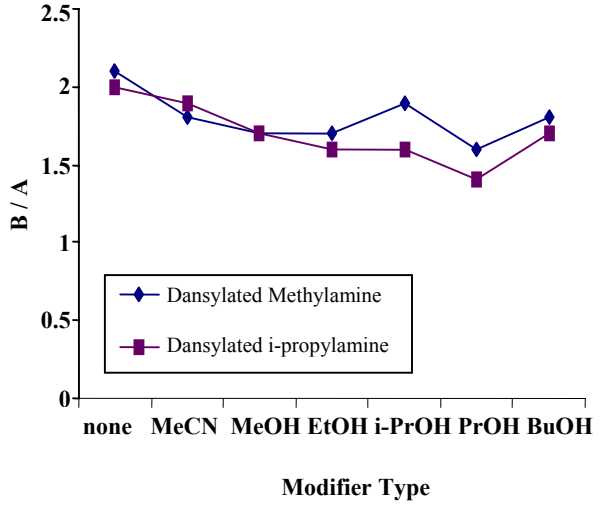


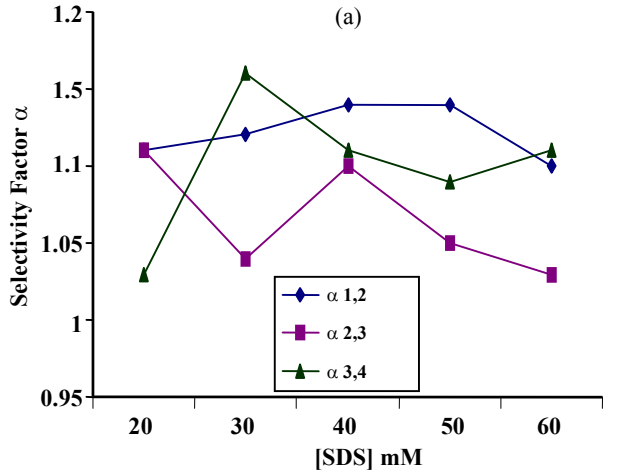

***Fig. 2: The effect of organic modifiers type on asymmetry factor. Conditions as Fig. 1.***

### *Selection the Type of organic modifier*

Addition of short-chain alcohols to the micellar mobile phase reduces the thickness of the film of surfactant molecules covering the stationary phase and thus results in enhanced efficiencies [15]. Furthermore, the presence of alcohol the micellar mobile phase, changes the retention mechanism by shifting the equilibrium of solutes from the stationary phase and micelle toward the bulk aqueous phase, which leads to reduction in the capacity factors [16]. Again two basic parameters N and B/A were investigated for selection of the best organic modifier. Results showed that a considerable improvement in efficiency is obtained by additing different organic solvents such as: acetonitrile, methanol, ethanol, 1-propanol, 2-propanol and butanol. Investigation of N and B/A showed that the appropriate modifier was 1-propanol (Figs. 1, 2).

### *The effect of surfactants concentration on capacity and selectivity factor*

Increasing the concentration of SDS and Brij-35 in the mobile phase increases the power of mobile phase and decreases the analysis time and the separation. In MLC plot of (1/K') vs. mixed micelle concentration is a straight line [6]. Considering the total analysis time, selectivity factor (Fig. 3) and peaks' shape, the appropriate SDS and Brij-35 concentration in mobile phase were found to be 40 mM and 0.5 mM respectively.



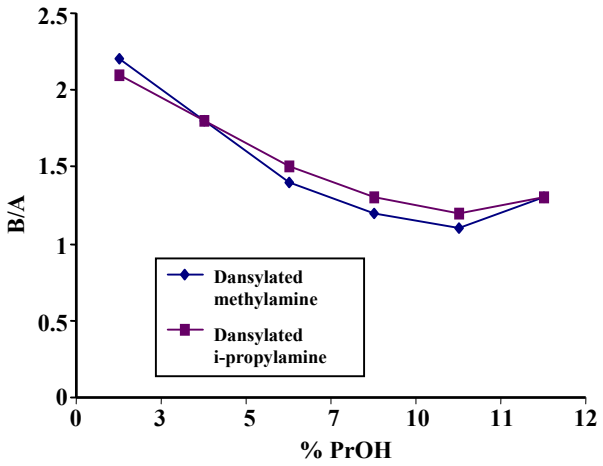




*Fig. 4: The effect of 1-propanol concentration on asymmetry factor. Conditions: mobile phase; 40 mM SDS + 0.5 mM Brij-35 + different percents of 1-propanol. Other conditions as Fig. 1.*

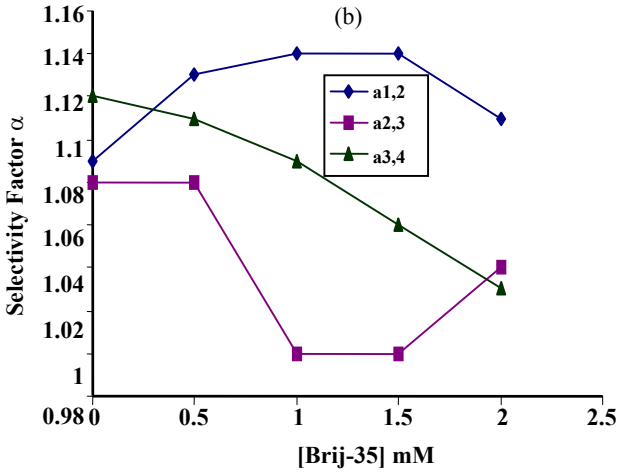



*Fig. 3: The effect of concentration of SDS and Brij-35 on selectivity factor Conditions: a) 20-60 mM SDS at [Brij-35]=0.5 mM + 5%(v/v) 1-PrOH; b) 0-2 mM Brij-35 + 40 mM SDS + 5%(v/v) 1-PrOH ; other conditions as Fig. 1. Samples: dansylated methylamine (1), ethylamine (2), morpholine (3) and isopropylamine (4).*

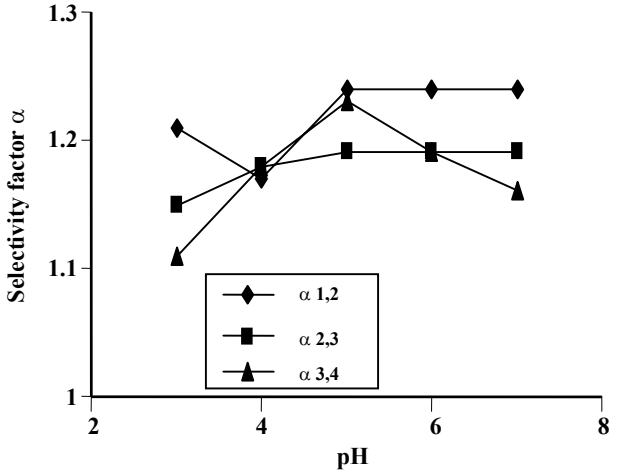



*Fig. 5: Variation of selectivity factor (α) against pH. Conditions: mobile phase; 40 mM SDS + 0.5 mM Brij-35 + 10% 1-propanol; flow rate 1.5 ml/min; column; $C_{18}$ (250×4.6 mm) 10μm, T=40°C .*
*Samples: dansylated methylamine (1), ethylamine (2), morpholine (3) and isopropylamine (4).*

### *The effect of 1-propanol concentration on capacity and selectivity factor*

A linear relationship usually exists between lnK´and the percent of organic modifier in the mixed MLC, at a constant micelle concentration [16, 17]. This effect for dansylated biogenic amines was also observed. To determine the appropriate amount of 1-propanol needed in the mobile phase to improve separation, parameters N and B/A were studied for two compounds. Results showed that addition of less than 12% 1-propanol improves peaks shape but the best efficiency was observed in 10% 1-propanol. The effect of propanol concentration on the selectivity factor (Fig. 4) also shows that 10% propanol was the best concentration for separation of dansylated biogenic amines.

### *The effect of mobile phase pH on retention and selectivity factor*

Retention of weak organic acids and bases is affected by the pH of the micellar mobile phase. Solute-micelle

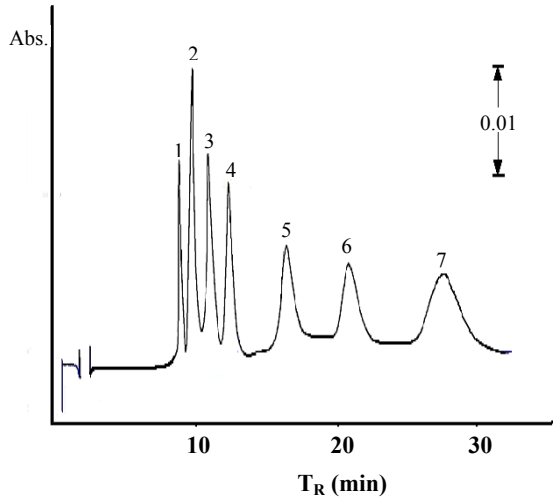



***Fig. 6: Chromatogram for separation of dansylated biogenic amines. Conditions: mobile phase; 40 mM SDS + 0.5 mM Brij-35 + 10% 1-propanol, pH=5, flow rate 1/5 ml/min, Column; $C_{18}$ (250×4.6 mm) 10µm, T=40°C. Samples: dansylated methylamine(1), ethylamine(2), morpholine(3), isopropylamine(4), buthylamine(5), diethylamine(6) and putresine(7).***

partition coefficients of the dissociated and undissociated forms of a compound are different. Changes in the pH can significantly alter the chromatographic retention, particularly when the mobile phase pH is close to the $pK_a$ value [18]. Dependence of k´ on the pH at a constant value of the micellar concentration is sigmoidal if there is no electrostatic repulsion between any of the two acid-base forms and surfactant molecules [19]. Variation of selectivity factor against pH (Fig.5) showed that the appropriate pH was about pH=5. The chromatogram of the dansylated biogenic amines in the mixed micellar mobile phase under appropriate conditions is shown in Fig. 6.

## CONCLUSIONS

A rapid, simple method for separation of biogenic amines was performed using the mixed micellar liquid chromatography. Since these compounds do not absorb light in the UV region, they were first derivatized by dancyl chloride and then analyzed by MMLC. In this way an appropriate separation time as well as a good resolution was also achieved. The optimum conditions were: 40 °C, 0.5 mM Brij-35, 40 mM SDS, 10% 1-propanol at pH 5.

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