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# A comparison of the TLC-densitometry and HPLC method for the determination of biogenic amines in fish and fishery products

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**Abstract**

A comparative study on the suitability of chromatographic techniques such as thin layer chromatography (TLC)-densitometry and high performance liquid chromatography (HPLC) for the analysis of biogenic amines in fish and fishery products was carried out. The HPLC method was found to offer a good linearity (5–100 ng), sensitivity and repeatability (<3%), but required sophisticated instrumentation, technical skill and high operational cost and time. On the other hand, with a little loss in linearity (20–300 ng) and repeatability (<8%), TLC-densitometry was found to be rapid and less expensive. In addition, this method is suitable for rapid and simultaneous screening of several samples at a time. Therefore, the TLC-densitometric method can be effectively used in the fish industry to detect biogenic amines, especially the toxic histamine, and putrescine and cadaverine, which can potentiate histamine toxicity in fish and fishery products. 

## 1. Introduction

Biogenic amines are formed in foods by the bacterial decarboxylation of free amino acids (Taylor & Sumner, 1987). They are of importance from the point of food intoxication and also as chemical indicators of spoilage. They are heat stable and therefore suitable for assessing the quality of heat-processed foods (Ababouch, Alaoui, & Busta, 1986; Ienistea, 1971). Detection of these amines in foods is often complicated because of the lack of a rapid detection method.

Several methods have been reported for the analysis of histamine and other amines, which include fluorimetric, enzymatic and chromatographic techniques. Among these, only the chromatographic techniques have the capacity to separate the different biogenic amines. Chromatographic techniques such as thin layer chromatography (TLC) (Chin & Koehler, 1983; Fleischer, 1979; Shalaby, 1994), gas liquid chromatography GLC; Starusckiewicz & Bond, 1981) and high performance liquid chromatography (HPLC) (Mietz & Karmas 1977; Yen & Hsieh, 1991) have been used for the analysis of biogenic amines. In the last few years, high resolution has become possible due to the advancements in the field of stationary phases for TLC. Precoated plates are commonly available and they can yield excellent separation in few minutes, and hence, a comparison was made between precoated TLC-densitometry and HPLC for the separation of biogenic amines in fish and fishery products. The factors taken into consideration are sensitivity, linearity, rapidity, repeatability, operational requirements, and efficiency.

## 2. Materials and methods

### 2.1. Biogenic amine analysis

#### 2.1.1. Preparation of standard amines

Standard putrescine, cadaverine, histamine, spermidine and tyramine were obtained from Sigma Chemicals Co., USA. A stock standard solution was prepared by diluting accurately 0.20–0.25 g of each compound in 10 ml of 5% trichloroacetic acid (TCA) solution. A 10-fold dilution of this solution formed the working standard.

#### 2.1.2. Derivatization of the amines

The amines were derivatized following the method of Rosier and Petegham (1988). One millilitre of the standard was taken in a 5-ml screw cap test tube, to which, 1.0 ml of phosphate buffer (pH 9.0, undiluted, E.Merck,

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India), a drop of 4 N sodium hydroxide solution and 2.0 ml of dansyl reagent (50 mg of dansyl chloride (Sigma grade) in 10 ml of acetone) were added. After thorough mixing, tubes were covered with aluminium foil and left in the incubator at 55°C for 1 h for dansylation. Tubes were cooled and stored at 5°C until further use.

### 2.2. TLC method

#### 2.2.1. Fractionation, detection and quantification of amines

The procedure of Fleischer (1979) was followed with slight modification. Amines were fractionated on precoated silica gel $GF_{254}$ TLC plate (0.25 mm thickness, 5×20 and 20×20, E. Merck, India). Ten microlitres of dansylated standard was applied on the plate and developed using chloroform:triethylamine (100:25) mixture. The plate was sprayed with isopropanol:triethanolamine (8:2) to enhance the fluorescence. Fractionated fluorescent amine spots were detected under the UV light at 365 nm. Amines were quantified by a computerized scanning densitometer (Model CS-930, Shimadzu Corporation, Kyoto, Japan) operated under the fluorescence mode (365 nm).

#### 2.2.2. HPLC method

Quantitative analysis of amines was also carried out on Waters HPLC system (Waters Associates, USA). Separation was achieved using a 300×3.9 mm, C-18 μ-Bondapak RP-column (Shandon HPLC, England) and a UV detector (Model-440) set at 254 nm. Solvents used were high purity HPLC grade methanol and water (vacuum filtered and degassed prior to use). The linear gradient elution programme described by Rosier and Petegham (1988) was followed. Dansylated amine standard (5–10 μl) was injected on to the column, after the equilibration with methanol/water (70/30) for 30 min. Methanol concentration was increased gradually to 75% after 3.5 min, 80% after 7.0 min, and finally, 100% after 10 min. A total separation time of 20 min was required for each sample and then the column was equilibrated. The instrument condition was adjusted to give a full-scale response by keeping AUFS at 0.1. Data were processed with a mircoprocessor-based integrator.

### 2.3. Statistical analysis

Linear regression coefficient and coefficient of variation between the volume applied and the area print-out were calculated following the standard procedures (Snedecor & Cochran, 1967).

### 2.4. Analysis of fish and fishery products for amines

Fresh, salt-cured and canned fish of a few varieties sold in the retail markets were analyzed for biogenic amines to study the nature and quantities of different amines. Fresh and canned fish (10 g) or salt-cured fish (5 g) were homogenized with 30 ml of 5% hot (80–90°C) TCA solution for 2 min and then, centrifuged at 3000 rpm for 10 min. Supernatant solution was filtered through Whatman No. 41 filter paper and 1 ml of the filtrate was used for derivatization.

## 3. Results and discussion

Extraction of amines from fish samples is an important step prior to the separation of biogenic amines. Extraction with 5% TCA solution offered rapid extraction (< 10 min) of amines. Earlier, Rosier and Petegham (1988) reported that an extraction time of 2 min seemed sufficient to reach a plateau. Using the TCA solution, a good recovery (98%) of the amines was also recorded by Luten et al. (1992). As amines are highly

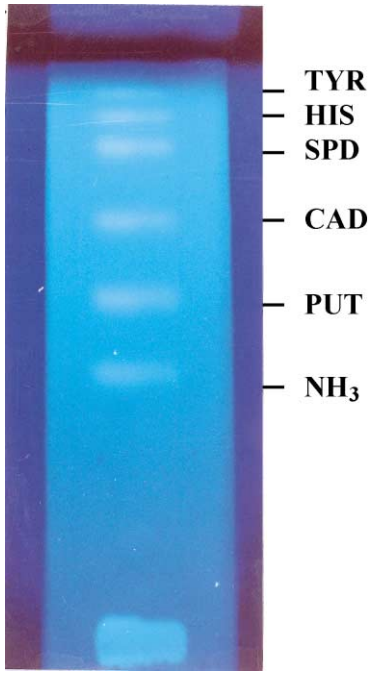


Figure 1. TLC separation of standard dansyl amines and ammonia on a precoated silica gel GF 254 plate: NH3 — Ammonia; PUT — Putrescine; Cad — Cadaverine; SPD — Spermidine; HIS — Histamine; TYR — Tyramine.

Table 1
Rf and Rt values of the different standard biogenic amines (dansyl derivatives) separated by TLC and HPLC techniques

| Amines | TLC (Rf values) | HPLC (Rt values) |
|---|---|---|
| Putrescine | 0.617 | 10.56 |
| Cadaverine | 0.724 | 11.24 |
| Spermidine | 0.824 | 16.23 |
| Histamine | 0.870 | 13.56 |
| Tyramine | 0.911 | 15.54 |

reactive substances, dansyl chlorides are used for derivatization of amines. The dansyl derivatives could be easily detected at a very low concentration under UV light due to their fluorescent characteristics. Dansyl chloride is also a non-specific reagent and was found to react with all the amino compounds such as amines, ammonia and free amino acids (Fleischer, 1979).

Precoated silica gel 60 GF plates were found to offer a neat and reproducible resolution of different amines. The order of separation of amines took place according to the increase in the molecular weight. Fig. 1 shows the TLC separation of different biogenic amines and Table 1 gives the Rf values. Dansyl amino acids and ammonia showed lower mobility, remained near the origin, and did not interfere with the separation of amines.

Detection of amines under the long wave UV light showed bright coloured fluorescent spots. Histamine appeared as yellowish, tyramine as green, and other amines as greenish blue spots. The minimum detection level was determined by the application of varied concentrations of different amines on the TLC plate. Detectable fluorescence spots and their response in the densitometer were observed; and the minimum detection level was found to be in the range of 15–20 ng. Fig. 2 shows the densitometric-scanning pattern of the standard amines and ammonia separated on the TLC plate. The order of separation of amines on TLC took place according to the increase in molecular weight. The distinctly resolved amine spots on the TLC plate were neatly scanned in the following order of separation viz., dansyl ammonia, putrescine, cadaverine, spermidine, histamine and tyramine as clear peaks.

Biogenic amines were neatly resolved using methanol and water under the gradient elution mode on HPLC (Fig. 3). The order of separation was different from that noticed in TLC. It was neither in increasing order of molecular weight nor basic nature. Table 1 gives the retention time (Rt) of the individual amines. The biogenic amines eluted as distinct peaks at different retention times. The interfering substances like ammonia and amino acids eluted much earlier than amines, as noticed in TLC. Amines could be detected at 5–10 ng levels by HPLC.

On TLC, it was possible to fractionate 10–12 samples simultaneously on one plate at a time by inserting two plates simultaneously and for development, 24 samples could be fractionated in 30–40 min. In the densitometer, time of scanning one sample was 10 min, whereas HPLC analysis required about 30 min (including equilibration time) for each sample and only one sample can be analysed at a time. Solvents required were of analar grade for TLC analysis and about 100 ml was sufficient for developing 24 samples. HPLC analysis required 30–

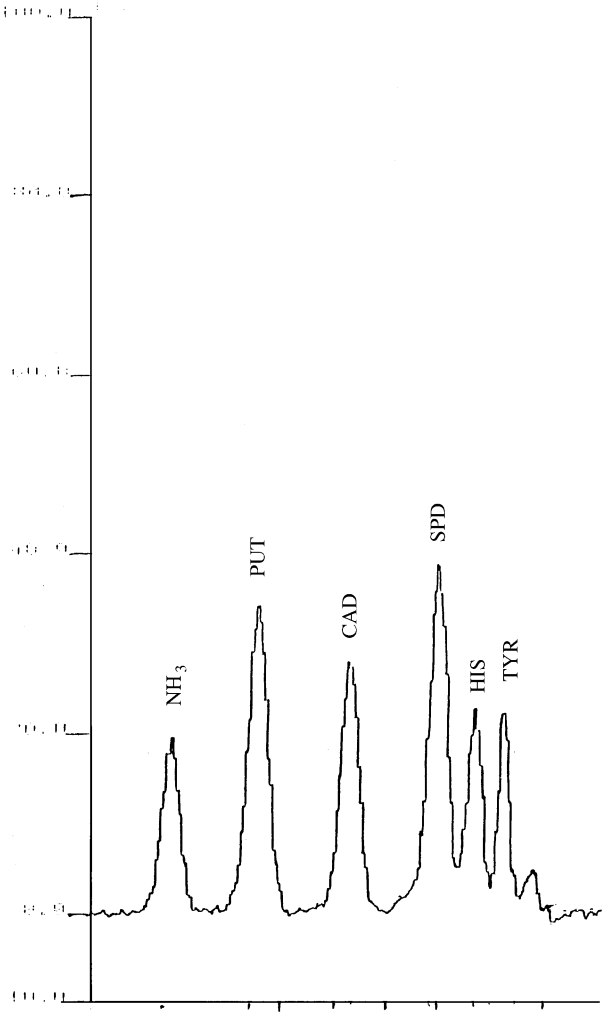


Fig. 2. TLC densitometric scanning pattern of standard dansyl amines and ammonia: NH3 — Ammonia; PUT — Putrescine; Cad — Cadaverine; SPD — Spermidine; HIS — Histamine; TYR — Tyramine.

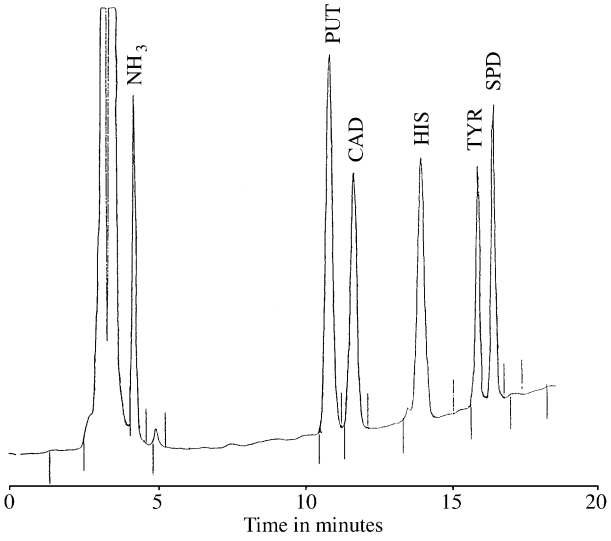


Fig. 3. HPLC separation of the standard dansyl amines and ammonia in a gradient elution programme: NH3 — Ammonia; PUT — Putrescine; Cad — Cadaverine; SPD — Spermidine; HIS — Histamine; TYR — Tyramine.

40 ml of high purity, particulate free special grade solvent for analysis of each sample, which is quite expensive. Therefore, TLC method of amine analysis was rapid and relatively inexpensive compared with the HPLC method, which required sophisticated instrumentation, careful maintenance, expensive solvents, accessories and high operational skill.

HPLC method is extensively used for the determination of biogenic amines in developed countries and it is reported to be more efficient, sensitive and reproducible compared to TLC. The linearity, sensitivity and repeatability of these methods were statistically examined and the results are given in Table 2. The response was linear over a range of 5–100 ng for HPLC and 20–300 ng for TLC. The sensitivity (fluorescent units per ng dansylated amine) of the amines on TLC was 4.94, 3.50, 0.84 and 0.37 for putrescine, cadaverine, histamine and tyramine, respectively. They were 12.30, 8.15, 9.35 and 5.80 for the HPLC method, in the same order. The linear regression coefficient ranged from 0.998–0.999 for HPLC and from 0.996–0.998 for TLC method, indicating only marginal differences between the two methods. The responses for amines were different in both methods because of the differences in the absorption characteristics and sensitivity. However, the quantities of amines determined by the two methods correlated well.

To test the repeatability, five different concentrations of each amine were analysed at least six times separately

Table 2
Linearity[a] and coefficient of variation for different amines analyzed by TLC and HPLC

| Amines | Regression coefficient ($r$) | Intercept (A) | Slope (B) | Linear equation | Coefficient of variation ($n=6$) |
|---|---|---|---|---|---|
| *Putrescine* | | | | | |
| TLC | 0.998 | −0.69 | 4.94 | $Y=-0.69+4.94\ X$ | 7.37 |
| HPLC | 0.998 | 4.27 | 12.30 | $Y=4.27+12.30\ X$ | 1.43 |
| *Cadaverine* | | | | | |
| TLC | 0.999 | −3.46 | 3.50 | $Y=-3.46+3.50\ X$ | 6.11 |
| HPLC | 0.997 | 2.96 | 8.15 | $Y=2.97+8.15\ X$ | 2.06 |
| *Histamine* | | | | | |
| TLC | 0.996 | −0.66 | 0.84 | $Y=-0.66+0.84\ X$ | 5.55 |
| HPLC | 0.999 | −0.27 | 9.35 | $Y=-0.27+9.35\ X$ | 2.87 |
| *Tyramine* | | | | | |
| TLC | 0.996 | −0.22 | 0.37 | $Y=-0.22+0.37\ X$ | 7.29 |
| HPLC | 0.998 | −1.90 | 5.80 | $Y=-1.90+5.80\ X$ | 2.80 |

[a] Fluorescence intensity on TLC and UV absorption intensity for HPLC with respect to increase in amine concentration. $Y$, Area print out by the Detector; $X$, ng of dansyl amines on TLC.

Table 3
Biogenic amine profile (in mg%) of some commercially important fresh canned and salt dried fish analyzed by TLC-densitometry and HPLC methods*

| Species | TLC-densitometry | | | | HPLC | | | |
|---|---|---|---|---|---|---|---|---|
| | Put | Cad | His | Tyr | Put | Cad | His | Tyr |
| *Fresh fish* | | | | | | | | |
| Mackerel | 0.95 | 2.80 | 2.07 | 2.58 | 0.82 | 3.23 | 1.95 | 2.74 |
| Sardine | 0.33 | 2.74 | ND[a] | 1.62 | 0.54 | 2.29 | ND | 1.18 |
| Seerfish | 1.42 | 1.77 | ND | 0.94 | 1.56 | 2.12 | ND | 1.07 |
| Shrimp | 1.14 | ND | ND | 0.88 | 1.34 | 0.41 | ND | 1.26 |
| *Canned fish* | | | | | | | | |
| Mackerel in brine | ND | 0.58 | ND | ND | 0.21 | 0.67 | ND | ND |
| Sardine in oil | ND | 0.17 | ND | ND | ND | 0.23 | ND | ND |
| Tuna in oil | ND | 0.18 | ND | ND | 0.11 | 0.19 | ND | 0.12 |
| *Salt-dried fish* | | | | | | | | |
| Mackerel | 26.44 | 95.59 | 32.10 | 39.84 | 30.18 | 97.62 | 35.26 | 41.38 |
| Sardine | 26.85 | 152.81 | 61.22 | 16.95 | 28.56 | 163.22 | 59.61 | 17.81 |
| Seerfish | 54.68 | 112.80 | ND | 15.42 | 56.65 | 124.80 | ND | 15.41 |
| Shrimp | 112.71 | 52.80 | ND | 70.47 | 110.55 | 55.32 | ND | 69.32 |

[a] ND, not detectable.

*Mean values of three determinations.

on TLC and HPLC. The good repeatability with a coefficient of variation of less than 8% was obtained for TLC, as observed earlier by some workers (Fleischer, 1979; Shalaby, 1994). However, the HPLC method offered a very good repeatability with a coefficient of variation of less than 3%.

To study the application of TLC densitometry and HPLC methods, the biogenic amine contents of fish and fishery products were determined separately by these methods, and the results are presented in Table 3. The quantities obtained by the two methods were more or less similar. The TLC densitometry method yielded slightly lower values for amines when compared to the HPLC method. However, the differences were not very significant.

The major amines detected in fish are putrescine, cadaverine, histamine and tyramine. In fresh and canned fish, the concentrations of these amines were relatively low and in a very few samples, histamine was detected in small amounts. The histamine concentration was far below the defect action level (DAL) of 10 mg% (Ababouch, 1991) and unlikely to cause toxicity problems. On the other hand, salt-cured fish contained relatively high amounts of biogenic amines, particularly in mackerel and sardine; histamine level was above the DAL. Earlier some workers have also recorded such high levels of histamine (Chakraborti, 1991; Subburaj, Karunasagar, & Karunasagar, 1984). The results elicit that despite a mariginal loss in linearity, repeatability and sensitivity, the TLC-densitometric method was also found to be an effective screening method for the determination of biogenic amines in fish and fishery products similar to that of HPLC.

In conclusion, for routine analysis of fish and fishery products, the TLC method using precoated silica plates coupled with densitometry can be used as a quick screening method to assess the presence of histamine and other potentiating amines. This method can be easily adopted by regulatory agencies and food industries as a qualitative tool for the simultaneous analysis of the quality as well as histamine toxicity hazards of seafoods.

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