Studies on the Determination of Biogenic Amines in Foods. 1. Development of a TLC Method for the Determination of Eight Biogenic Amines in Fish

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Ingestion of foods that contain high levels of histamine causes clinical symptoms, namely histamine poisoning. During the course of this investigation eight biogenic amines in fish samples, including histamine, putrescine, cadaverine, tyramine, tryptamine, spermine, spermidine, and β-phenylethylamine, were separated and quantitated by TLC technique. Many solvent systems were evaluated, with the best separation in one dimension when benzene/triethylamine (5:1) was used as a developing system, which enabled separation of histamine in addition to spermine, tyramine, and β-phenylethylamine. However, the other four biogenic amines were not separated using this developing system. The only way to separate the eight tested biogenic amines was by using a two-dimensional TLC procedure. The running system in the first direction was benzene/triethylamine/acetone (10:2:1), and the solvent system in the second direction was benzene/triethylamine (5:1). This method provides a simple and inexpensive means to separate and detect biogenic amines in fish samples.

Keywords: *Biogenic amines; histamine poisoning; scombroid poisoning; thin layer chromatography (TLC); dansyl chloride; spectrofluorometer*

INTRODUCTION

Biogenic amine accumulation in foods usually results from decarboxylation of amino acids by enzymes of bacterial origin. Histamine is a well-known toxic substance characterized by severe symptoms. Other biogenic amines such as cadaverine and tyramine were considered to be histamine potentiators. Ingestion of foods that contain high levels of histamine and other biogenic amines causes clinical symptoms, namely histamine poisoning (Taylor, 1983; Eitenmiller and De-Souza, 1984).

Different methods were used for biogenic amine determination. These methods are summarized as follows: (a) Bioassay methods depend on the observations of guinea pig ileum contraction (Barsoum and Gaddum, 1935). (b) Colorimetric assay used a ninhydrin indicator, and the density of color was measured spectrophotometrically. Also, Kimata (1961) used Pauly's diazo reagent, and the absorbance at 510 nm was determined by spectrophotometry. (c) A fluorometric assay was based on conversion of amine to a stable and strongly fluorescing product by reaction with a suitable reagent such as dansyl chloride. The current official method (AOAC, 1975) used *o*-phthalaldehyde for derivatizing histamine (Shore *et al.*, 1959); the resulting fluorescence is measured by spectrophotofluorometer or densitometer. (d) An enzymatic isotopic assay was based on the incubation of tissue samples with tracer amounts of (^{3}H)histamine and (^{14}C)-S-adenosylmethionine in the presence of the enzyme histamine methyltransferase (Arnold and Brown, 1978). (e) Gas–liquid chromatography, by which histamine, cadaverine, and putrescine were determined in foods using their perfluoropropionyl derivatives, was performed on a 3% OV-225 column held at 180 °C with electron capture detector (Staruszkiewicz and Bond, 1981). (f) High-pressure liquid chromatography (HPLC) is a new technique that allows simultaneous analysis of histamine and other biogenic amines in foods. A μBondapak (R) column (Waters, Inc.), a C_{18} column, a UV detector (254 nm), and a solvent system of 0.02 M acetic acid plus acetonitrile (9:1) and 0.02 M acetic acid plus acetonitrile plus methanol (2:9:9) were used for biogenic amines determination in foods (Mietz and Karmas, 1978). (g) Radioimmunoassay (RIA), a method for amine determination, has not yet been applied to food samples; however, it is based on the development of a specific antibody for histamine, and once antibodies are raised, the possibility of development of a simple and easily applicable enzyme-linked immunoassay (ELISA) is feasible (Taylor, 1983).

Through this study, we develop a simple TLC technique that can be efficiently used to determine all of the major biological active amines that may be responsible for scombroid poisoning.

MATERIALS AND METHODS

A. Materials. During handling of standard materials and suspect samples, the following precautions should be taken: 1, wear gloves; 2, avoid mouth pipetting; 3, prepare the developing system in a ventilation system; 4, assume fish samples to be contaminated until proven otherwise; and 5, safely dispose of contaminated glassware.

1. Sample Preparation. Sardine fish was obtained from a local market, and the flesh tissue was separated and cut into small pieces.

TLC aluminum sheets (20 × 20 cm) and silica gel 60 without fluorescence indicator, layer thickness 0.2 mm (E. Merck), were used. Histamine–2HCl, putrescine–2HCl, cadaverine–2HCl, tyramine–HCl, tryptamine–2HCl, spermine, spermidine, and β-phenylethylamine (Sigma) were used for preparing stock standard solutions (0.5 mg/mL).

Dansyl chloride (Sigma), 500 mg, was dissolved in 100 mL of acetone.

2. Solvent System Preparation. Ten developing solvent systems were suggested for biogenic amine separation as follows: benzene/triethylamine (TEA) (5:1); ethyl acetate (EA)/cyclohexane (65:35); benzene/triethylamine (10:1); benzene/triethylamine/ammonia solution (5:1:1); benzene/triethylamine/methanol (10:1:2); benzene/acetone (10:2); benzene/triethylamine/acetone (10:2:1); benzene/methanol (10:2); chloroform

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Table 1. R_f Values of the Tested Biogenic Amines[a] Using Different Solvent Systems

		R_f values							
no.	developing system	putr	cadav	spd	tryp	spm	hist	tyr	β-phe
1	benzene/TEA (5:1)	0.06	0.08	0.09	0.09	0.13	0.20	0.33	0.41
2	EA/cyclohexane (65:35)	0.40	0.48	0.35	0.45	0.30	0.22	0.45	0.55
3	benzene/TEA (10:1)	0.04	0.05	0.07	0.05	0.10	0.15	0.28	0.34
4	benzene/TEA/ammonia solution (5:1:1)	NS[a]	NS	NS	NS	NS	NS	NS	NS
5	benzene/TEA/MeOH (10:1:2)	0.45	0.46	0.50	0.42	0.53	0.51	0.55	0.55
6	benzene/acetone (10:2)	NS	NS	NS	NS	NS	NS	NS	NS
7	benzene/TEA/acetone (10:2:1)	0.30	0.34	0.36	0.41	0.24	0.37	0.48	0.50
8	benzene/MeOH (10:2)	NS	NS	NS	NS	NS	NS	NS	NS
9	chlo/2-propanol (25:1)	NS	NS	NS	NS	NS	NS	NS	NS
10	chlo/MeOH/ammonia solution (2:2:1)	NS	NS	NS	NS	NS	NS	NS	NS

[a] NS, no separation.

(chlo)/2-propanol (25:1); and chloroform/methanol/ammonia solution (2:2:1).

B. Methods. *1. Extraction of Prepared Samples.* The prepared samples were extracted according to a modification of the method described by Mietz and Karmas (1978) [using 25 g of prepared sample and 125 mL of 5% trichloroacetic acid (TCA) instead of 50 g of prepared sample in 225 mL of 5% TCA]. No adverse effect on the accuracy of the method was found as reported by Ayesh (1992).

2. Extraction of Biogenic Amines. Twenty-five grams of homogenized fish flesh was blended with 125 mL of 5% TCA for 3 min using a Waring blender. After centrifugation, the clear layer was completed to 125 mL with 5% TCA. Ten milliliters of the extracts was transferred to a suitable culture tube with 1 mL of 50% NaOH and 4 g of NaCl with shaking and extraction by 1-butanol/chloroform (1:1 v/v) (3 × 5 mL). An equal volume of *n*-heptane was added to the combined organic phase; then, the biogenic amines were extracted by several 1 mL portions of 0.02 N HCl, and the aqueous extract was evaporated at 95 °C with a current of air.

3. Preparation of Dansylamines. Derivative formation using dansyl chloride for amine standards and sample extracts were performed according to the method of Mietz and Karmas (1978).

4. Dansylation of Biogenic Amines. The final extract of biogenic amines was mixed with 1 mL of dansyl chloride and 0.5 mL of saturated sodium bicarbonate. After at least 10 h, about 15 mL of distilled water was added. Then the dansylamines were extracted by three portions (5 mL) of diethyl ether. The ether extract was evaporated. The residue was diluted in a suitable volume with acetonitrile.

5. Recovery Determination. About 5 mg of each amine was added to 25 g of fish samples (sardine) which had different amounts of these amines. Extraction, separation, and quantitation procedures were done as described above.

6. Preparation of TLC Plates. Glass plates (20 × 20 cm) were coated with a 250 μm layer of silica gel G 60, using a slurry consisting of 30 g of silica gel suspended in 70 mL of distilled water. Plates were dried at 100 °C for 90 min and stored immediately in a desiccator until used. The plates were activated at 100 °C for 30 min before use.

7. Separation of Dansylamines. a. One-Dimensional TLC. Ten microliters of standard dansylamines was spotted 2 cm from the base of the plates with capillary pipets or a microsyringe. The dansylated compounds were separated by ascending developing systems. After chromatography was performed, the fluorescent dansyl derivative zones were visualized and were marked by using a UV light source (360 nm), and the R_f values of dansylated amines were calculated (Table 1).

b. Two-Dimensional TLC. Two straight lines were inscribed on the plate parallel to two contiguous sides (2 cm from each side), to limit migration of the solvent front (Figure 1). The following solutions were spotted on the plate using a capillary pipet as follows: at point A, 10 μL of the dansylated extract or mixture standard; at points B and C, 10 μL of the dansylated mixture standard.

The plate was developed in the first direction in solvent system 7 [benzene/triethylamine/acetone (10:2:1)] until the solvent front reached the solvent limit line. The plate was taken out of the jars and allowed to dry in the dark. Then,

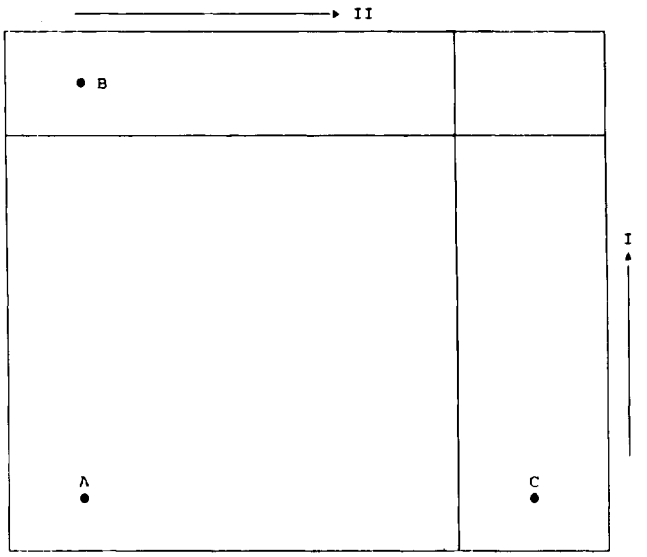

Figure 1. Schematic diagram showing the position of both dansylated extract and standard spots: (A) sample extract; (B, C) standard mixtures; (I) benzene + triethylamine + acetone (10+2+1); (II) benzene + triethylamine (5 + 1).

the plate was turned 90° and developed in the second direction in the second solvent system [no. 1 (benzene/triethylamine (5:1))]. The plate was allowed to dry at room temperature, and then it was dried by using a hair dryer until the excess solvent disappeared.

8. Quantitation. The resulting zones were examined under longwave ultraviolet light. The dansylamine areas were marked. The silica gel containing the dansylamine was scraped off, extracted with 3 mL of acetonitrile, and centrifuged. The clear solution was quantitated by spectrophotofluorometer (Aminco-Bowman spectrophotofluorometer, American Instrument Corp., Silver Spring, MD).

9. Standard Curves. The standard curve for each dansylamine was developed concurrently on a separate TLC plate. These were processed in the same manner with the exception that they were developed only with the benzene/triethylamine (5:1) solvent.

RESULTS AND DISCUSSION

A. Separation. Data in Table 1 show the R_f values calculated during the separation of mixture of the eight dansylamines [putrescine (putr), cadaverine (cadav), histamine (hist), tyramine (tyr), tryptamine (tryp), spermine (spm), spermidine (spd), and β-phenylethylamine (β-phe)] by the suggested 10 solvent systems using the one-dimensional TLC technique. It is clear from the data that solvent systems 4, 6, and 8–10 showed no separation.

However, solvent systems 1–3, 5, and 7 showed different levels of separation depending on the tested dansylamines. Only two compounds could be isolated

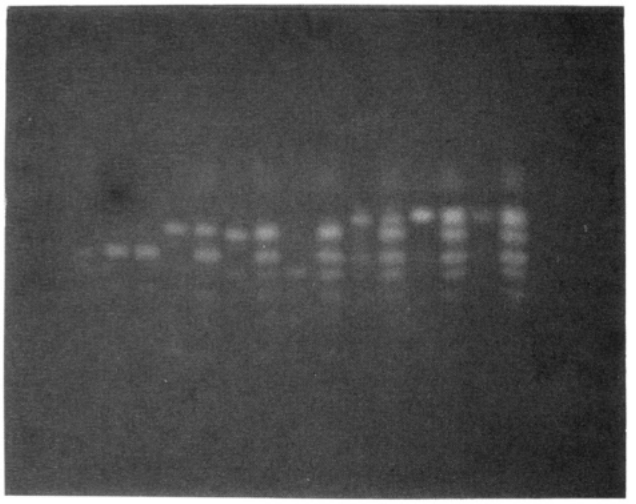

Figure 2. TLC separation of the tested dansylamines using benzene/triethylamine/methanol (10:2:1) in one dimension.

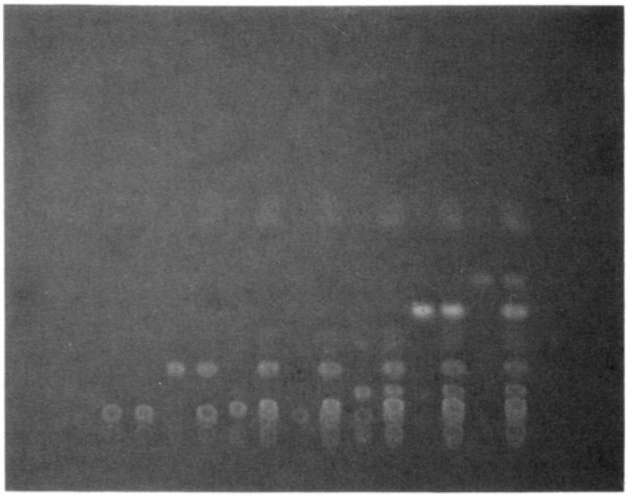

Figure 3. TLC separation of the tested dansylamines using benzene/triethylamine (5:1) in one dimension.

using solvent systems 2, 3, and 5—histamine and β-phenylethylamine with both systems 2 and 3 and tryptamine and spermine with system 5 (Figure 2). More dansylamines were separated, namely histamine, spermidine, tyramine, and β-phenylethylamine, through system 1 [benzene/triethylamine (5:1)] as shown in Figure 3. Thus, the best separation was achieved by using solvent system 7 [benzene/triethylamine/acetone (10:2:1)], which separated six of the eight tested compounds (putrescine, cadaverine, spermidine, tryptamine, spermine, histamine, tyramine, and β-phenylethylamine) (Figure 4).

On the other hand, by adopting the two-dimensional TLC procedure, it was possible to separate the eight tested dansylamines. The running system in the first direction was benzene/triethylamine/acetone (10:2:1), and the solvent system in the second direction was benzene/triethylamine (5:1) (Figures 5 and 6).

B. Quantitation. Scanning for excitation and emission wavelengths was performed for all of the individually tested dansylamines (Table 2). The different fluorescence units recorded versus the different concentrations of all the examined dansylamines are given in Table 3. Linear relationships between fluorescent units and the concentrations of the tested dansylamines were observed within the concentrations 0.5–3.7 μg. These relationships are illustrated in Figures 7 and 8, and the

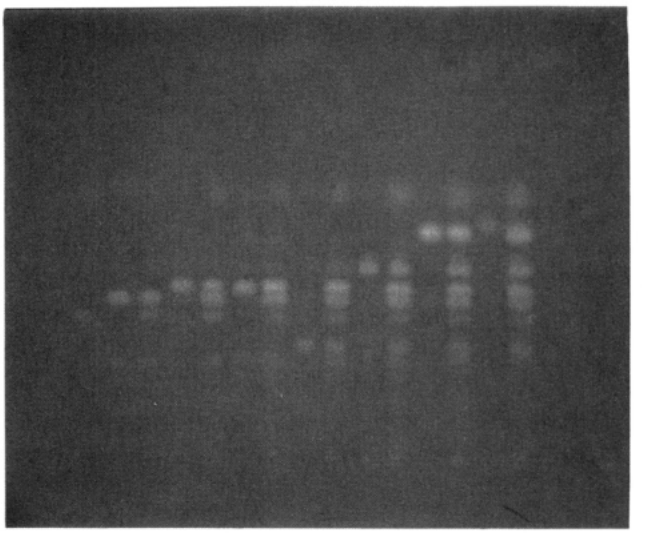

Figure 4. TLC separation of the tested dansylamines using benzene/triethylamine/acetone (10:2:1) in one dimension.

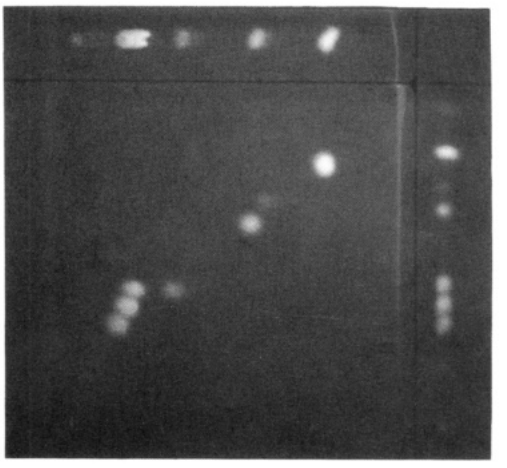

Figure 5. TLC separation of the tested dansylamines using (I) benzene/triethylamine/acetone (10:2:1) and (II) benzene/triethylamine (5:1) in two dimensions.

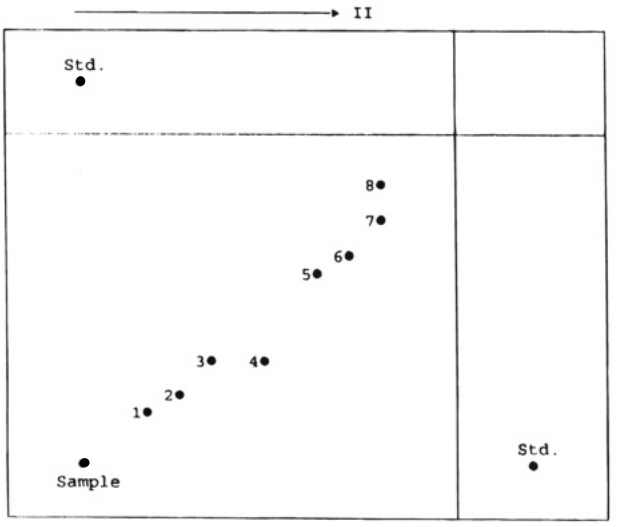

Figure 6. Schematic diagram for the position of the tested dansylamines using a two-dimensional technique: (I) benzene + triethylamine + acetone (10+2+1); (II) benzene + triethylamine (5+1); (1) putrescine; (2) cadaverine; (3) tryptamine; (4) spermidine; (5) spermine; (6) histamine; (7) tyramine; (8) β-phenylethylamine.

Y-axis intercept (*a*), the slope (*b*), and the correlation coefficient (*R*) were recorded.

Table 2. Excitation and Emission Wavelengths for the Tested Dansylamines

substrate (dansylamine)	wavelength (nm) excitation	emission
putrescine	340	560
cadaverene	335	570
spermidine	370	530
tryptamine	360	520
spermine	350	520
histamine	360	520
tyramine	360	520
β-phenylethylamine	340	510

Table 3. Relations between the Examined Dansylamine Concentrations and the Fluorescent Units

concn (μg)	fluorescent unit of putr	cadav	spd	tryp	spm	hist	tyr	β-phe
0.5	0.30	0.15	0.70	0.20	0.40	0.10	0.20	0.40
1.0	0.60	0.35	1.50	0.40	0.80	0.15	0.40	0.90
1.5	0.90	0.50	2.10	0.60	1.30	0.20	0.60	1.80
2.5	1.40	1.00	3.50	1.00	2.00	0.30	1.00	2.10
3.75	2.20	1.55	5.00	1.55	3.20	0.40	1.50	3.40

Data presented in Table 4 show the recovery (percent) of the development TLC method using fish samples (sardine), which had different amine contents. Results show that all samples contained cadaverine and tryptamine; however, histamine was detected in only one sample, while putrescine was detected in three samples. Concerning the recovery percent, the recorded averages of the tested amines were 93.62, 91.96, 88.00, 86.96, 84.56, 84.04, 81.18, and 77.92 for tryptamine, tyramine, putrescine, cadaverine, spermine, histamine, spermidine, and β-phenylethylamine, respectively. Also, it was noticed that the minimum recovery value was recorded in β-phenylethylamine (77.92%), while the maximum value (93.62) was observed in tryptamine. The obtained results were considered satisfactory with regard to the number of the determined components (eight amines). In this respect Staruszkiewicz (1977) compared histamine determination by the fluorometric method and by the AOAC colorimetric method and reported that the fluorometric method has been adopted as official first action. Also, Vidal-Carou *et al.* (1990) determined histamine in fish and meat by spectrofluorometric method and reported that this method is reliable for single histamine determination.

It should be noted that our development method is reliable for the determination of eight biogenic amines, if necessary, in one sample by two-dimensional TLC; at the same time, this method is suitable for determination of at least 4 amines in more than 10 samples using one-dimensional TLC.

Commercially prepared plates (20 × 20 cm, E. Merck) were used to compare the separation of the tested biogenic amines between this kind of plate and the "home-made" silica gel plates. No difference in the quality of the separation has been noticed within the used biogenic amine concentrations (Table 3). Also, no difference in the developing time (35 min at 20 °C) was noticed between the two kinds of plates.

From this presentation the following main points could be concluded:

1. Solvent system 1 [benzene/triethylamine (5:1)] is suitable for the detection of histamine, spermidine, tyramine, and β-phenylethylamine using the one-dimensional TLC as a simple, rapid, and economic technique for detection of four biogenic amines including

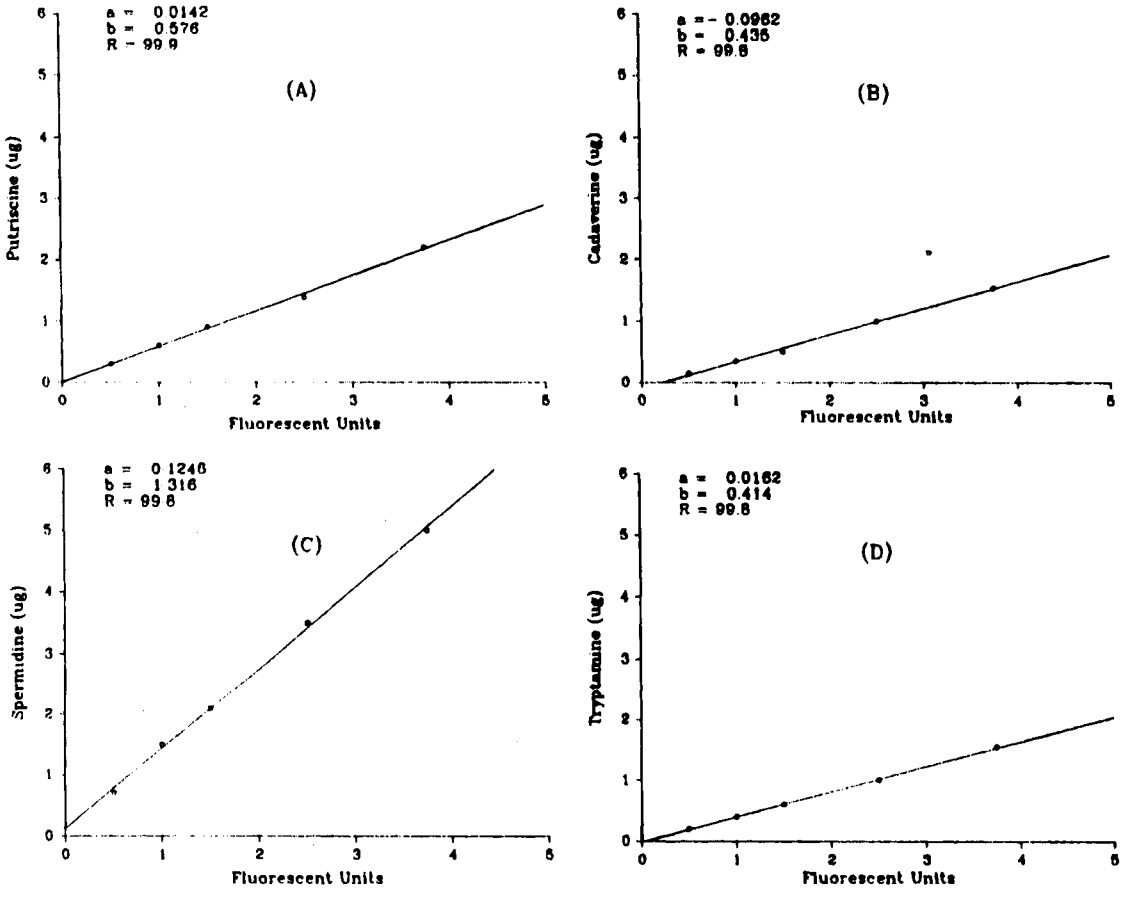

Figure 7. Relations between the concentrations of dansylamines (μg) and the fluorescent units for putrescine (A), cadaverine (B), spermidine (C), and tryptamine (D): a = intercept; b = slope; R = correlation coefficient.

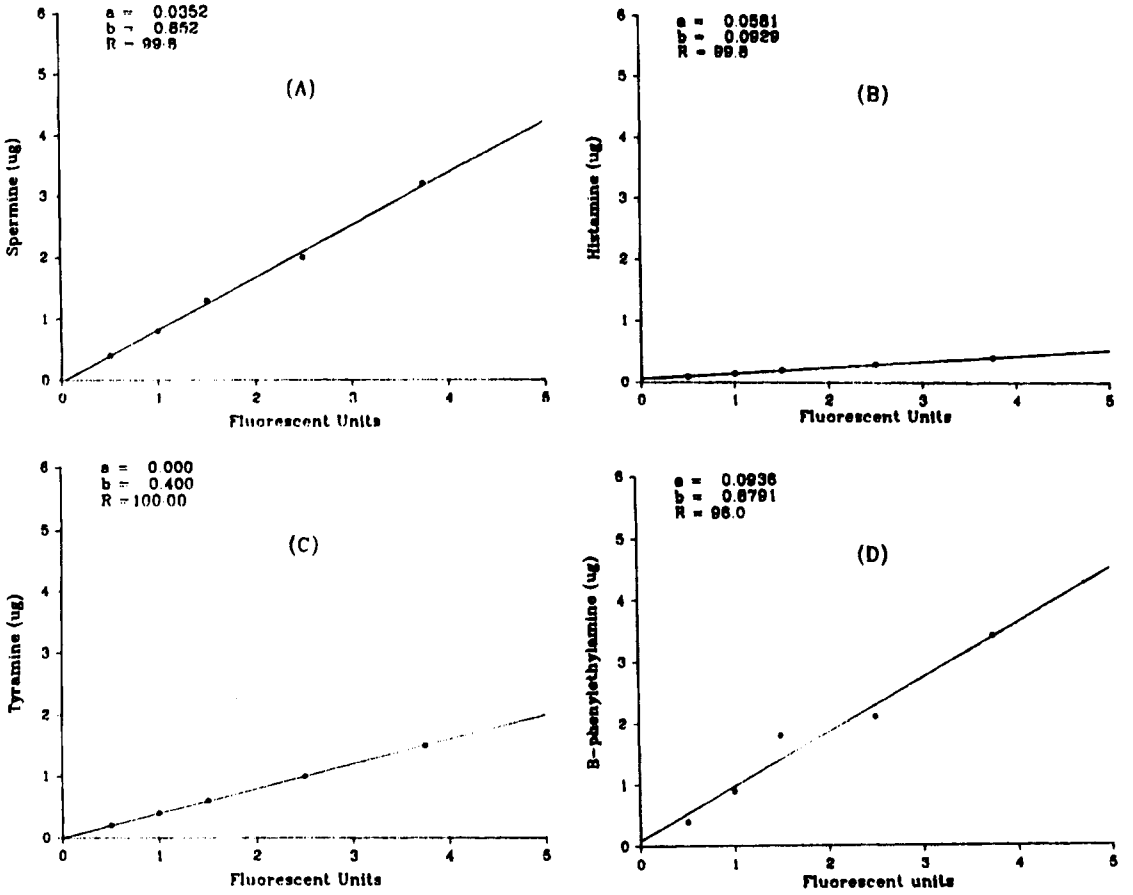

Figure 8. Relations between the concentrations of dansylamines (μg) and the fluorescent units for spermine (A), histamine (B), tyramine (C), and β-phenylethylamine (D): a = intercept; b = slope; R = correlation coefficient.

Table 4. Recovery of the Studied Method for Fish Samples with Different Amine Contents

	sample										
	I		II		III		IV		V		
amine	BG[a]	R (%)[b]	BG	R (%)	BG	R (%)	BG	R (%)	BG	R (%)	av % ± SD
histamine	0	83.0	30.0	86.2	0.0	84.5	0	84.0	0	82.5	84.04 ± 1.44
tyramine	0	91.6	100.0	90.6	0.0	93.2	0	90.1	0	94.3	91.96 ± 1.76
cadaverine	8	87.5	3.2	85.7	14.5	78.0	5	88.1	12	86.5	86.96 ± 0.92
putrescine	75	91.3	0.0	86.2	0.0	85.9	9	87.4	9	89.2	88.00 ± 2.26
spermine	0	86.7	0.0	81.6	0.0	84.0	0	83.4	0	87.1	84.56 ± 2.32
spermidine	0	85.1	0.0	78.2	0.0	77.6	0	82.3	0	82.6	81.18 ± 3.19
tryptamine	62	93.1	38.0	95.2	50.0	93.7	25	90.8	25	95.3	93.62 ± 1.84
β-phenylethylamine	0	77.8	0.0	72.6	0.0	97.1	0	78.8	0	81.3	77.92 ± 3.24

[a] BG, background (μg/100 g). [b] R (%), recovery (%).

histamine, the major factor in histamine or scombroid poisoning.

2. Solvent system 7 [benzene/triethylamine/acetone (10:2:1)] is a good system for the separation of six dansylamines in one-dimensional TLC. The main defect of this solvent system is the interference between histamine and spermine, which means that this system is not recommended for the detection of histamine.

3. The best separation and detection of the eight tested biogenic amines could be reached by using two-dimensional TLC with two solvent systems, benzene/triethylamine/acetone (10:2:1) in the first direction and benzene/triethylamine (5:1) in the second direction.

In conclusion, it is worth mentioning that different methods were used for biogenic amine determination. The bioassay methods for biogenic amines in foods have not yet been applied to food samples. The radioimmunoassay method uses tritiated histamine and requires a liquid scintillation counter, and it may not be widely applicable in food testing laboratories (Taylor, 1983). The colorimetric assay is considered not entirely satisfactory (Taylor, 1983).

In addition, Lark and Bell (1976) reported that the official method (AOAC, 1975) is slow and time-consuming and lacks sensitivity at very low levels; it is used only for histamine determination.

High-pressure liquid chromatography (HPLC), gas–liquid chromatography, and enzymatic isotopic assay are among the more recent techniques. These techniques offer some advantages in accuracy and flexibility, yet there are some difficulties in their availability, cost, and experience. The apparatus needed are usually available to only the most sophisticated analytical laboratories due to their higher costs. They also require special experience for operators.

On the other hand, the developed TLC technique that was reached through this study can assay numerous samples with a minimum of time, effort, and cost. Also,

it is simpler and more efficient and has satisfactory accuracy. Finally, it can be available in all food testing laboratories.

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