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Separation, identification and estimation of biogenic amines in foods by thin-layer chromatography

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A method for the separation, identification and estimation of eight biogenic amines (histamine, cadaverine, putrescine, phenylethylamine, tyramine, tryptamine, spermidine and spermine) using silica gel TLC and spectrophotofluorometry is described. The complete resolution of the dansylated derivatives of amines could not be achieved by one-dimensional TLC using any of 12 solvent systems examined. However, the derivatives could be well separated by two-dimensional TLC in which the first development system was benzene/triethylamine/acetone (10 : 1 : 2, v/v/v), while in the second direction the development system was benzene : triethylamine (5 : 1 v/v). The R_f values and the fluorescent colours of the amine dansyl derivatives aided their identification. The separated fluorescent derivatives were extracted with acetonitrile and estimated spectrophotofluorometrically. The results obtained indicated that the method is sensitive and precise, where the relative standard deviation was less than 10%. The method was applied to 10 dry sausage samples and 10 fish samples. Dry sausage contained the eight biogenic amines with an average concentration similar to that which had been previously reported for sausage or meat products. Fish samples were free from tyramine, tryptamine and phenylethylamine. The other amines were found to be variable in their concentrations.

INTRODUCTION

Toxaemias resulting from the ingestion of foods containing biologically active amines have been reviewed (Smith, 1981; Stratton *et al.*, 1991). Biologically active amines which have physiological effects in humans are generally either psychoactive or vasoactive. Psychoactive amines affect the nervous system by acting on neural transmitters, while vasoactive amines act on the vascular system (Lovenberg, 1973).

Biologically active amines (histamine, putrescine, cadaverine, tyramine, tryptamine, phenylethylamine, spermine and spermidine) have been found in many foods such as fish and fish products (Taylor, 1985; Yamanaka, 1990; Yen and Hsieh, 1991), cheese and fermented meat products (Stratton *et al.*, 1991).

Several methods to isolate and estimate amines have been reported. Reagents such as perchloric acid (Koehler and Eitenmiller, 1978; Yen, 1986), trichloroacetic acid (Zee *et al.*, 1983), hydrochloric acid (Rice *et al.*, 1975) and organic solvents (Redmond and Tseng, 1979) are commonly used for the extraction of amines from food. Several biogenic amines often occur simultaneously in these extracts, so that analytical procedures that permit the separation and estimation of these compounds in the same aliquot of an extract are desirable. In this regard, various analytical techniques have been developed for the separation of biogenic amines. Paper chromatography (Herbst *et al.*, 1958; Perry and Schroeder, 1963), thin layer chromatography (Chin and Koehler, 1983), gas liquid chromatography (Kaplan *et al.*, 1974; Staruszkiewicz and Bond, 1981) and high performance liquid chromatography (Mietz and Karmas, 1978; Yen, 1986) have all been advocated for the separation and identification of amines or amine derivatives. However, interference by several unidentified factors has often necessitated the inclusion of additional chromatographic separation steps (Ough, 1971; Voigt *et al.*, 1974; Lerke and Bell, 1976).

The objective of this study is to develop a simple TLC method for the separation, identification and estimation of eight dansylated amines (i.e. histamine, putrescine, cadaverine, tyramine, tryptamine, phenylethylamine, spermine and spermidine) in foods.

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MATERIALS AND METHODS

Chemicals and supplies

Amines (histamine 'Him', cadaverine 'Cad', putrescine 'Put', tyramine 'Tyr', tryptamine 'Try', B-phenylethylamine 'B-phe', spermine 'Spm' and spermidine 'Spd') as their crystalline hydrochlorides, dansyl chloride (5-dimethylaminonaphthalene-l-sulphonyl) and thin-layer chromatography (TLC) plates (20 × 20 cm aluminium sheets precoated with 0·20 mm silica gel G-60) were purchased from Merk. The standards were prepared at a concentration of 0·5 mg ml^{-1}.

All chemicals were of reagent grade. Double-distilled water and solvents were used.

Source of samples

Food samples (sausage and fish) were obtained from local markets in Cairo, Egypt.

Extraction of amines

Ground food (50 g) was extracted with 5% trichloroacetic acid (TCA) (3 × 75 ml) using a Waring Blender. Each blended mixture was centrifuged and the clear extracts combined. The volume was adjusted to 250 ml with 5% TCA solution. The equivalent of 2 g of samples as the TCA extract (about 10 ml) was made alkaline with sodium hydroxide and extracted with *n*-butanol/chloroform (1 : 1 v/v) (3 × 5 ml). The combined organic phase, after the addition of an equal amount of *n*-heptane, was extracted with several 1 ml portions of 0·02 N HCl and the aqueous extract taken to dryness.

Derivative formation

The dansylated derivatives of the amines were formed by adding saturated sodium bicarbonate solution (0·5 ml) to the residue, then adding dansyl chloride reagent (500 mg in 100 ml acetone) (1 ml) using a Vortex mixer while adding the reagent. After standing for more than 10 h, the dansylamines were extracted by adding water and extracting the mixture with several portions of diethyl ether. The combined ether extracts were evaporated to dryness and the residue redissolved in acetonitrile (2 ml).

Standard solution preparation

Three mixtures of standard solutions were prepared, in addition to each amine separately. The first one (8 MS) contained the eight amines, the second (4 MS1) contained four amines (tyramine, tryptamine, spermidine and spermine), and the third one (4 MS2) contained the other four amines (histamine, cadaverine, putrescine and phenyl ethylamine). Of each amine solution (0·5 mg ml^{-1}) (200 μl) were used. Using a current of air and a steam bath, the prepared solutions were evaporated to less than 200 μl. The dansyl derivatives were prepared as described above. The residues were dissolved in 5 ml acetonitrile (10 μl = 0·2 μg of amine as derivative).

Separation of the dansylamines

1 One-dimensional thin-layer chromatography

The chromatographic separation was carried out to evaluate 12 solvent systems (Table 1) for their ability to separate the eight dansylamines, using dansylated standard solutions. Thin-layer chromatography plates were activated 1 h at 110°C before use. The standard amine derivatives were applied 2 cm from the base of the plates with quantitative capillary pipettes. The dansylated compounds were separated by ascending development for 15 cm. After chromatography was complete, the fluorescent dansyl derivative zones were visualized and marked with the aid of a suitable UV-light source (360 nm) and the R_f values were calculated.

2 Two-dimensional thin-layer chromatography

Two straight lines were inscribed on the plate parallel to two contiguous sides (3 cm from each side), to limit migration of the solvent fronts (Fig. 1). The following

Table 1. Solvent systems compared for their ability to separate dansylated amines

Solvents	Ratio	References
(A) Systems reported for the separation of amines:		
1. Chloroform : methanol : ammonia	2 : 2 : 1	Lieber and Taylor (1978)
2. Methanol : ammonia	20 : 1	Lieber and Taylor (1978)
3. Acetone : ammonia	95 : 5	Lieber and Taylor (1978)
(B) Systems reported for the separation of dansylamines:		
1. Benzene : triethylamine	5 : 1	Chin and Koehler (1983)
2. Chloroform : triethylamine	5 : 1	Fleisher and Russell (1975)
3. Ethylacetate : cyclohexane	65 : 35	Spinelli *et al.* (1974)
(C) Suggested systems for the separation of dansylamines:		
1. Benzene : triethylamine	10 : 1	
2. Benzene : triethylamine : methanol	10 : 1 : 1	
3. Benzene : triethylamine : methanol	10 : 1 : 2	
4. Benzene : triethylamine : acetone	10 : 1 : 1	
5. Benzene : triethylamine : acetone	10 : 1 : 2	
6. Benzene : acetone	5 : 1	

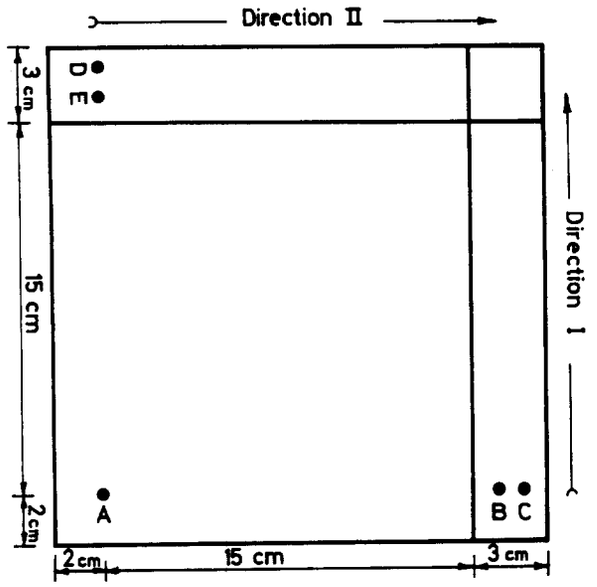

Fig. 1. Schematic of thin-layer chromatogram for two-dimensional chromatography A, spotting place for sample extract or mixture standards (8MS); B,E, spotting places for mixture standards (4MS2); C,D, spotting places for mixture standards (4MS1).

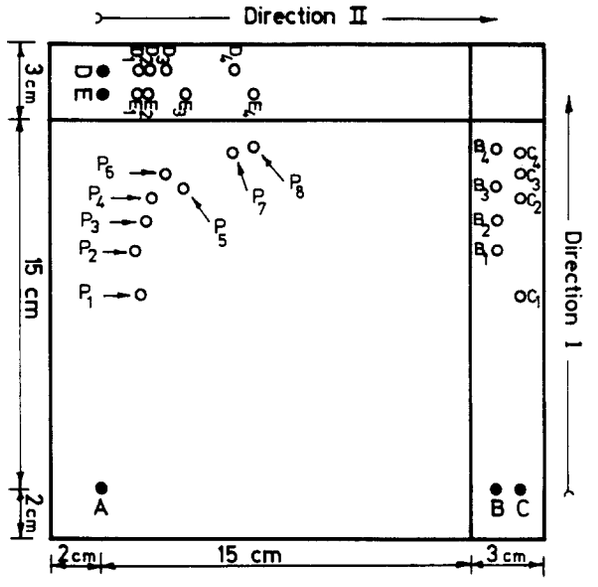

Fig. 2. Schematic of thin-layer chromatogram after two-dimensional development. (B_1, E_1, locations of dansylcadaverine spots originating from 4MS2 applied at B and E respectively; B_2,E_2, locations of dansylputrescine spots originating from 4MS2 applied at B and E respectively; B_3, E_3, locations of dansylhistamine spots originating from 4MS2 applied at B and E respectively; B_4, E_4, locations of dansylphenylethylamine spots originating from 4MS2 applied at B and E respectively; C_1, D_1, locations of dansyltryptamine spots originating from 4MS1 applied at C and D respectively; C_2, D_2, locations of dansylspermidine spots originating from 4MS1 applied at C and D respectively; C_3, D_3, locations of dansylspermine spots originating from 4MS1 applied at C and D respectively; C_4, D_4 locations of dansyltyramine spots originating from 4MS1 applied at C and D respectively; P_1–P_8, locations of dansylamines, spots originating from sample or 8MS applied at A).

solutions were spotted on the plate using capillary pipette:

— At point A, 10 μl of the dansylated extract or 8 MS solution.
— At points B and E, 10 μl of the dansylated 4 MS1 solution.
— At points C and D, 10 μl of the dansylated 4 MS2 solution.

The plate was developed in the first direction in benzene : triethylamine : acetone (10 : 1 : 2) until the solvent front reached the solvent limit line. The plate was taken out of the jar and allowed to dry in the dark. Then, the plate was turned through 90° and developed in the second direction in benzene : triethylamine (5 : 1), in an equilibrated tank, to the pencil line. The plate was allowed to dry at room temperature, then dried with a hair dryer until the excess of solvent disappeared before interpretation.

N.B. Benzene is a highly toxic solvent and great care must be exercised with its use. Alternatively toluene may be substituted for benzene, but the separation may not be identical.

Interpretation of the chromatogram

The chromatogram was examined under long-wave (360 nm) ultraviolet light. Each dansylated amine had a special fluorescence. Two imaginary lines were projected from the position of each standard (B_1, B_2, B_3 and B_4 with D_1, D_2, D_3 and D_4, and C_1, C_2, C_3 and C_4 with E_1, E_2, E_3 and E_4) and at right angles to the development direction (Fig. 2). The intersections (P_1 – P_8) of these lines are the locations in which to expect to find the dansylamine spots originating from the tested portion extract or standards mixture (8 MS) applied at A.

Quantitation

The areas of dansylamines were marked and the silica gel containing the dansyl derivatives was scraped off, extracted with acetonitrile (5 ml), and centrifuged. The clear solution was estimated by spectrophotofluorometer (Aminco-Bowman SPF). The blank zone from the same plate was removed and extracted in the same manner establishing the fluorescence zero point. The wavelengths that gave maximum sensitivity were used for excitation and emission for each dansylated amine and are given in Table 2.

The standard curves were obtained for each dansylated amine on separate TLC plates. They were processed in the same manner with one exception in that they were developed only with benzene : triethylamine (5 : 1, v/v) in an equilibrated tank.

RESULTS AND DISCUSSION

Separation and identification of amines

An initial visual inspection, under UV, of the chromatograms obtained (one-dimensional TLC) in the solvent systems listed in Table 1 indicated that systems

Table 2. Excitation and emission wavelengths of the dansyl derivatives of polyamines

Substance	Wavelengths (nm)	
	Excitation	Emission
Putrescine	340	560
Cadaverine	335	570
Spermidine	330	530
Tryptamine	360	520
Spermine	350	520
Histamine	360	520
Tyramine	360	520
B-phenylethylamine	340	510

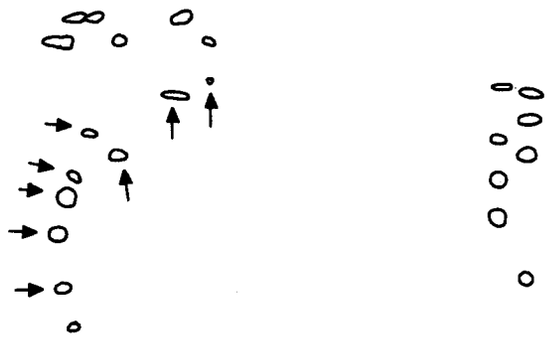

Fig. 3. Two-dimensional separation of dansylated biogenic amines of sausage sample. For description of spots, see Fig. 2.

B–1, B–3, C–1, C–3 and C–5 were likely candidates for separation of the tested dansylated biogenic amines on silica gel G plates. Other combinations of solvents did not achieve satisfactory separation. There are a number of chromatographic systems reported for the separation of polyamines (Table 1(A)). None of these systems, however, was effective for the separation of the dansylated derivatives which we examined. A few developing systems were reported to separate the dansylated amines (Table 1(B)), however, none of these was satisfactory for the large number of derivatives examined. The suggested solvent systems (Table 1(C)), were also unreliable as satisfactory developing systems for the separation of the tested derivatives. However, the chromatograms obtained (one-dimensional TLC) in the five potential systems indicate that each separated spot was symmetrically shaped and did not tail.

The R_f values of the dansylated derivatives of the polyamines using the potential solvent systems are listed in Table 3. Using system B–1, Put, Try, Cad and Spd were very closed to each other (R_f = 0·09, 0·10, 0·12 and 0·13 respectively), on the other hand, Spm, Him, Tyr and B-phe were well separated. When system B–3 was applied, interference between Try and Tyr (R_f = 0·45) was noticed. Moreover, Put and Cad (R_f = 0·40 and 0·42 respectively) were not clearly separated. System C–1 led to interference between Cad and Try (R_f = 0·05), while Put and Spd were very close to each other (R_f = 0·04 and 0·07, respectively). With system C–3, Tyr, Put and Spd interfered with the detection of B–phe, Cad and Him, respectively. Using system C–5, satisfactory separation of Spm, Cad, Spd, Try and Tyr (R_f = 0·85, 0·73, 0·78, 0·52 and 0·91 respectively) was obtained. However, Spd somewhat interfered with detection of Him (R_f = 0·78 and 0·80, respectively) and B-phe interfered with the detection of Tyr (R_f = 0·92 and 0·91, respectively).

From the results obtained the solvent system C–5 is the best developing system tested. The defect of this system was the interference of Him with Spd and Tyr with B-phe. This problem can easily be solved by developing the chromatogram obtained in the second direction using solvent system B–1 which led to good separation of Him from Spd, and Tyr from B-phe. The separation pattern of the eight dansylated amines (extracted from sausage) using two dimensional TLC was photographed and is presented in Fig. 3.

It is clear that the dansylated biogenic amines were well separated and easily identified. Dansyl chloride is a suitable reagent for the analysis of amines (Seiler and Wiechman, 1970; Seiler, 1971) since it gives rapid quantitative reaction with amines under mild reaction conditions in aqueous media. An important quality of the dansyl derivatives in the identification and determination of amines is their excellent chromatographic behaviour (Chin and Koehler, 1983) which led to the separation obtained (Fig. 3). The characteristic fluorescent colours of the dansylated amines as well as the R_f values (Table 3) were useful in their identification. Therefore, the chromatographic method mentioned here is recommended for the analysis of samples which contain large numbers of amines.

Table 3. R_f values and fluorescent colours of the dansyl derivatives of polyamines

Substance	R_f values					Fluorescent colour
	B–1[a]	B–3	C–1	C–3	C–5	
Putrescine	0·09	0·40	0·04	0·45	0·64	Green
Tryptamine	0·10	0·45	0·05	0·42	0·52	Greenish yellow
Cadaverine	0·12	0·42	0·05	0·46	0·73	Green
Spermidine	0·13	0·35	0·07	0·50	0·78	Green
Spermine	0·17	0·30	0·10	0·53	0·85	Green
Histamine	0·23	0·22	0·15	0·51	0·80	Orange-yellow
Tyramine	0·37	0·45	0·28	0·55	0·91	Yellow
B-phenylethylamine	0·42	0·55	0·34	0·55	0·92	Greenish yellow

[a] Refers to solvent systems of Table 1.

Table 4. Linearity of fluorescence intensity for increasing concentrations of dansylamines on TLC

Amines	Determination coefficient (r^2)	Intercept (A)	Slope (B)	Equation
Try	0·999	0·041	0·435	Y=0·041 + 0·435X
Tyr	0·999	0·069	0·457	Y=0·069 + 0·457X
Put	0·994	0·034	0·581	Y=0·034 + 0·580X
Cad	0·999	0·003	0·294	Y=0·003 + 0·294X
Spm	0·998	0·016	0·878	Y=0·016 + 0·878X
Spd	0·999	0·031	1·341	Y=0·031 + 1·341X
Him	0·986	0·026	0·105	Y=0·026 + 0·105X
B-phe	0·989	0·015	0·891	Y=0·015 + 0·891X

X=μg of dansylamines on TLC.
Y=response of spectrophotofluorometer (fluorescent unit).

Estimation of amines

A standard curve was prepared for each amine derivative after TLC separation, elution and fluorometric measurement. The results are given in Table 4. It is clear that the response of the spectrophotofluorometer was linear and highly correlated with the amounts of amine derivatives, where the calculated coefficient (r^2) ranged from 0·999 to 0·986, and each dansylated amine had own linear equation. The sensitivity (fluorescent units per μg dansylated amine) of the dansylated amines were 0·435, 0·457, 0·581, 0·294, 0·878, 1·341, 0·105 and 0·891 for Try, Tyr, Put, Cad, Spm, Spd, Him and B-phe, respectively.

To investigate the repeatability, two amounts of each dansylated amine (1·25 μg and 3·0 μg, respectively) were analysed ten times. The results are summarized in Table 5. A relative standard deviation RSD (ISO, 1981) of less than 10% was obtained for all amines tested. A maximum RSD of 9·41% was reported for spermine when 3 μg was applied on TLC, while the minimum RSD (1·13%) was obtained for 1·25 μg tyramine applied on TLC.

Table 5. Precision of the TLC method for analysis of amines

Substrate	Amounts applied (μg)	Detected amounts (μg)		Standard deviation	Relative standard deviation
		Range	Mean		
Try	1·250	0·94–1·29	1·124	0·017	1·15
	3·00	2·66–3·35	2·986	0·128	4·29
Tyr	1·250	1·05–1·49	1·239	0·014	1·13
	3·00	2·48–3·35	2·912	0·175	6·01
Put	1·25	1·06–1·32	1·181	0·042	5·86
	3·00	2·52–3·13	2·936	0·105	6·03
Cad	1·250	1·01–1·35	1·231	0·105	8·53
	3·000	2·71–3·22	2·966	0·155	5·23
Spm	1·250	1·12–1·41	1·235	0·082	6·64
	3·000	2·83–3·29	2·954	0·278	9·41
Spd	1·250	1·10–1·36	1·237	0·084	6·79
	3·000	2·81–3·11	2·975	0·092	3·09
Him	1·250	0·99–1·47	1·229	0·031	2·52
	3·00	2·13–3·75	2·991	0·248	8·29
B-phe	1·250	1·11–1·33	1·218	0·086	7·06
	3·000	2·68–3·35	2·974	0·193	6·99

Table 6. Biogenic amines content of dry sausage samples

Amines	Positive samples (%)	Concentrations (μg g^{-1})	
		Range	Average
Cadaverine	100	12·50–56·25	33·44
Putrescine	100	6·56–26·25	13·31
Tyramine	90	0·00–15·88	6·75
Tryptamine	70	0·00–26·25	6·10
Spermidine	50	0·00–6·00	1·50
Spermine	50	0·00–2·75	0·97
Histamine	20	0·00–15·00	2·10
Phenylethylamine	10	0·00–30·00	3·00

The method was used to determine the biogenic amine content of dry sausage and fish samples. Concerning dry sausage data (Table 6), histamine was found in 20% of the tested sausage samples. The average concentration was 2·1 μg g^{-1} (0–15 μg g^{-1}). The histamine values reported here are close to those reported previously for dry sausage by Taylor *et al.* (1978), Rice *et al.* (1975), Cantoni *et al.* (1974) and Henry (1960). Putrescine and cadaverine were found in all dry sausage tested, the average concentrations were 33·44 and 13·31 μg g^{-1}, respectively. Similar results were obtained for beef, pork, lamb and ham (Lakritz *et al.*, 1975; Edwards *et al.*, 1983). Tyramine was found in 90% of the tested sausage with an average of 6·75 μg g^{-1}. The average value was lower than that previously reported by Koehler and Eitenmiller (1978), Rice *et al.* (1975) and Vandekerckhove (1977). Tryptamine was found in 70% of the tested sausage samples with an average of 6·1 μg g^{-1}. Phenylethylamine was not found in many sausage samples, the average level was 3 μg g^{-1}, presented only in 10% of the tested dry sausage. The polyamines (spermine and spermidine) were found in 50% of the tested sausage samples, with an average of 0·97 and 1·5 μg g^{-1}, respectively.

The method was also used to determine the biogenic amine content of 10 samples of bolti fish, the data obtained are given in Table 7. Fish samples were free from tyramine, tryptamine and phenylethylamine. Histamine was found in 80% of the tested samples with an average of 9·52 μg g^{-1} the other four amines, i.e. cadaverine, putrescine, spermidine and spermine were found in all samples tested, the corresponding averages

Table 7. Biogenic amines content of fish samples

Amines	Positive samples (%)	Concentrations (μg g^{-1})	
		Range	Average
Cadaverine	100	0·2–15·50	9·12
Putrescine	100	0·6–5·50	3·13
Tyramine	0	—	—
Tryptamine	0	—	—
Spermidine	100	0·6–2·50	1·60
Spermine	100	0·5–3·25	1·73
Histamine	80	0·0–27·00	9·52
Phenylethylamine	0	—	—

of their presence were 9·12, 3·13, 1·60 and 1·73 $\mu g\ g^{-1}$. Remarkable differences were observed in the concentration of amines in all fish samples tested. However, all the levels recorded were relatively low. Differences in enzymatic activity and the bacterial flora involved in fish samples may be related to the complex situation associated with the production of the biogenic amines (Schulze and Zimmermann, 1982). However, the low concentrations of biogenic amines recorded here in fish samples could be due to the fact that this fish species (Bolti) is a non-scombroid fish and is not associated with scombroid poisoning (Taylor *et al.*, 1984).

From the aforementioned results, it could be concluded that the method described offers a simple but effective and accurate means for the separation and estimation of biogenic amines and that it should be applicable to foods in general.

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