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THIN-LAYER CHROMATOGRAPHIC SCREENING METHODS FOR HISTAMINE IN TUNA FISH*

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SUMMARY

Twelve solvent systems were tested for their ability to separate histamine and histidine on a variety of thin-layer coatings. The best solvent–adsorbent systems were: chloroform–methanol–ammonia (2:2:1), methanol–ammonia (20:1), acetone–ammonia (95:5), and double development with (a) *n*-butanol–acetone–water (2:2:1) and (b) chloroform–methanol–ammonia (12:7:1), all on silica-gel layers. Ninhydrin was used as the visualization reagent. These four systems were then evaluated for their potential use as rapid screening procedures in the detection of possibly deleterious levels of histamine in tuna fish. Successful separation of histamine from the other ninhydrin-positive components of methanolic tuna fish extracts was achieved with all four systems. A sample from a lot of tuna implicated in human illness was found to have a histamine level considerably higher than tuna purchased from a local retail outlet or an extract spiked to a histamine level considered to be a threshold value for toxicity symptoms. The methanol–ammonia (20:1) and chloroform–methanol–ammonia (2:2:1) systems, used with silica-gel plates, are the most promising for rapid preliminary screening of tuna fish extracts for histamine.

INTRODUCTION

Among the many food-borne hazards which have been implicated in a number of outbreaks of food poisoning is histamine[1–7], although relatively few cases of histamine poisoning have actually been reported. Apparently the small amounts of histamine usually present in foods pose little hazard to the consumer. At the present time, a histamine concentration of 100 mg per 100 g food sample is considered to be the critical level for histamine poisoning[2,4–7].

Histamine in foods results from the microbial decarboxylation of histidine. Consequently, those foods which originally contained large amounts of histidine and

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which have been exposed to microbial degradation or fermentation may contain sufficient histamine to cause occasional food poisoning episodes. The foods most often implicated in such outbreaks have been fish of the suborder *Scombroidei*, namely tuna, mackerel, bonita, albacore and skipjack[1,2,7–15]. As a result, histamine analysis has now become a routine quality control procedure, especially in the tuna fish processing industry. Such quality control procedures require analysis of large numbers of samples, most of which would contain histamine at levels far below that which would cause symptoms of toxicity.

Even the simplified method for histamine analysis developed in this laboratory[16] is not conducive to rapid, routine screening of large numbers of samples simultaneously. For this reason, a number of thin-layer chromatographic (TLC) methods were examined for their potential utility as rapid, preliminary, semi-quantitative screening methods for the detection of histamine in tuna fish extracts. Those samples exhibiting a higher than normal histamine level could then be analyzed by the more accurate fluorometric assay[16].

EXPERIMENTAL

Chemicals and Supplies

Amino acid, amine and dipeptide standards were obtained from the following sources: histamine dihydrochloride, L-lysine monohydrochloride, glycine and L-tryptophan from J. T. Baker (Phillipsburg, N.J., U.S.A.); L-histidine, L-carnosine, cadaverine, agmatine sulfate, D,L-octopamine hydrochloride, β-phenylethylamine, putrescine, serotonin (5-hydroxytryptamine), spermidine trihydrochloride, spermine tetrahydrochloride, thiamine hydrochloride, trimethylamine hydrochloride, tyramine and tryptamine hydrochloride from Sigma (St. Louis, Mo., U.S.A.); and L-histidyl-L-leucine and L-histidyl-L-serine from Vega-Fox Biochemicals (Tucson, Ariz., U.S.A.). Ninhydrin (reagent grade) was also obtained from J. T. Baker. All other chemicals and solvents used were reagent grade.

Pre-coated thin-layer plates were purchased as follows: Avicel, MN 300 Cellulose-Normal, MN 300 Cellulose-CM and silica gel G plates, all without fluorescent indicator, from Analtech (Newark, Del., U.S.A.); Permakotes from Applied Science Labs. (State College, Pa., U.S.A.); and EM silica gel plates (without fluorescent indicator) from VWR Scientific (San Francisco, Calif., U.S.A.).

Cans of chunk light tuna in oil (sample A) and fancy albacore solid white tuna in water (sample B) were purchased in a local supermarket. A sample of canned chunk light tuna in oil, from a lot of which was known to have caused illness (sample C), was generously supplied by Dr. Harold S. Olcott, University of California, Davis, U.S.A.

Methods

All amino acid, amine and dipeptide standard spotting solutions were prepared in methanol at 2 mM concentrations. If the standard was difficult to dissolve in methanol alone, enough water was added to effect solution.

Initially a variety of adsorbents and solvent systems were tested for their histamine–histidine separation properties. The solvent systems used for these separations are listed in Table I.

TABLE I

SOLVENT SYSTEMS COMPARED FOR THEIR ABILITY TO SEPARATE HISTAMINE AND HISTIDINE

System No.	*Solvents*	*Reference*
I	*n*-Butanol–acetone–water (2:2:1)	
II	Chloroform–methanol–conc. ammonia (12:7:1)	17
III	Double development; first in I, then II	
IV	Chloroform–methanol–conc. ammonia (2:2:1)	18*
V	Methanol–conc. ammonia (20:1)	19
VI	Acetone–conc. ammonia (95:5)	20
VII	Ethyl acetate–glacial acetic acid–water (3:3:2)	17
VIII	*n*-Butanol–glacial acetic acid–water (4:1:1)	18
IX	*n*-Butanol–acetone–diethylamine–water (10:10:2:5)	21
X	Isopropanol–88% formic acid–water (20:4.5:5)	21**
XI	Isopropanol–88% formic acid–water (20:1.5:5)	21**
XII	*sec.*-Butanol–methyl ethyl ketone–dicyclohexylamine–water (10:10:2:5)	21

* Original reference used 17% ammonia.
** Original reference used 99% formic acid.

Ten microliters of sample were applied to plates which had been prewashed in reagent-grade acetone. The spots were allowed to air-dry before placing the plates in the developing chamber. The layers on 20-cm plates were scored across the top at a point 16 cm from the origin. This procedure straightened any irregularities in the solvent front and prevented the plate from overdeveloping.

Ten grams of each well-mixed tuna sample were extracted with methanol according to the method described by Taylor *et al.*[16]. The undiluted methanol extract obtained after centrifugation was used as the spotting solution for TLC. In addition, a 1:10 dilution of the extract of sample C was made with methanol. Quantitative analysis of the three tuna samples used in this study by the method noted above[16] showed the histamine levels to be: (1) sample A, chunk light tuna in oil, 10.7 mg/100 g tuna; (2) sample B, albacore solid white tuna in water, 3.5 mg/100 g tuna; and (3) sample C, chunk light tuna in oil, 300 mg/100 g tuna. The methanol extract of sample B was spiked with histamine to a level of 64 mg/100 g tuna. Such a level approaches the threshold histamine concentration which can give rise to clinical symptoms of toxicity[2,4–7].

After development all plates were either air-dried or gently warmed on a hot plate until all residual solvent was gone. Spots were visualized with a ninhydrin spray containing 300 mg ninhydrin in 100 ml *n*-butanol to which was added 3 ml glacial acetic acid[22]. Plates developed with a system containing ammonia were warmed 1–2 min on a hot plate immediately before spraying with ninhydrin to reduce any background reaction. After spraying the plates were again warmed 1–2 min on the hot plate to speed spot development.

A check of the sensitivity of this ninhydrin reagent for the detection of histamine was done on EM silica-gel plates developed in solvents IV and V. The 2-m*M* histamine standard was diluted 1:10 and 1:100 with methanol, and 5–40 μl spots of each dilution were applied to the plate. In order to eliminate spreading of the spots,

TABLE II

COMPARISON OF REPRESENTATIVE R_F VALUES OF HISTAMINE AND HISTIDINE OBTAINED USING DIFFERENT COMBINATIONS OF ADSORBENTS AND SOLVENT SYSTEMS

The R_F values are those obtained on a single chromatogram and, therefore, must be regarded only as guide values.

Adsorbent	*Source*	*Compound*	*Solvent systems compared*											
			I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
Avicel	Analtech	Histamine	0.20*,**	—	—	—	—	—	—	—	0.85*	0.55*,**	0.36, 0.23*,***	—
		Histidine	0.14	—	—	—	—	—	—	—	0.51	0.37	0.23	—
MN-300 cellulose-Normal	Analtech	Histamine	0.33, 0*,***	—	—	—	—	—	—	—	0.95*	0.69*	0.26*,**	—
		Histidine	0.13	—	—	—	—	—	—	—	0.57	0.55**	0.20	—
MN-300 cellulose-CM	Analtech	Histamine	—	—	—	—	—	—	—	—	—	—	—	0.19
		Histidine	—	—	—	—	—	—	—	—	—	—	—	0.58
Silica gel G	Analtech	Histamine	0.06	0.46	0.50	0.77	0.39	0.85	0.24	0.03	0.71*	0.24*,**	0.36*,**	0.04
		Histidine	0.31**	0.15	0.39	0.72	0.67	0.05	0.32	0.03	0.71	0.24**	0.29	0.48
Silica gel	EM	Histamine	0	0.37	0.39	0.67	0.33	0.60	0.18	0.04	—	—	—	—
		Histidine	0.08	0.14	0.14	0.61	0.82	0	0.28	0.05	—	—	—	—
Silica gel permakotes	Applied Science Labs.	Histamine	—	—	—	0.96	0.92	0.96	—	—	—	—	—	—
		Histidine	—	—	—	0.92	0.96	0.10	—	—	—	—	—	—

* These TLC plates were microscope slides coated with the appropriate adsorbent. All other separations were accomplished on 20 cm plates.

** Tailing.

*** Two spots were visible.

repeated applications of 10 μl of the diluted standard were made, with complete drying of the spot between applications, until the desired volume had been applied. The amount of histamine applied per spot ranged from 100 pmoles to 20 μmoles.

RESULTS AND DISCUSSION

Comparison of R_F values of histamine and histidine (Table II) indicated that systems III, IV, V and VI were likely candidates for the separation of histamine in tuna extracts on silica gel plates. Other combinations of solvent and adsorbent also achieved effective separations; however, such factors as tailing, very high or low R_F values for histamine, long development time or residual solvent that was difficult to remove, also influenced the choice of adsorbent and solvents listed over the others.

The silica gel G layers supplied by Analtech had a slightly shorter development time than the EM silica gel plates. In addition, both histamine and histidine had higher R_F values on the Analtech plates. However, the surface of the EM plates used for these comparisons was less disturbed during sample applications than that of the Analtech plates which were available in the laboratory. For this reason, EM plates were chosen for all subsequent experiments as a matter of convenience.

The data presented in Table III indicate that in these four systems, histamine is well separated from other amino acids, biogenic amines and dipeptides. Low levels of putrescine, cadaverine and histamine, along with high levels of spermine and spermidine, have been found in unspoiled tuna[23]. As the tuna decomposes, however, the levels of these compounds are completely reversed. Comparison of the R_F values

TABLE III

REPRESENTATIVE R_F VALUES OF AMINE STANDARDS

R_F values are those obtained on a single chromatogram and, therefore, must be regarded only as guide values.

Amine	*Solvent system*			
	III	*IV*	*V*	*VI*
Histamine	0.32	0.78	0.35	0.43
Agmatine	0.02	0.15	0.03	0.21
Cadaverine	0.05	0.28	0.62	0.30
Carnosine	0.07	0.58	0.06	0
Glycine	0.22	0.62	0.64	0
Histidine	0.18	0.70	0.75	0
Histidyl–leucine	0.41	0.72	0.86	0
Histidyl–serine	0.17	0.64	0.84	0
Lysine	0.03	0.40	0.21	0
Octopamine	0.53	0.78	0.62	0.67
β-Phenylethylamine	0.81	1.00	0.58	0.77
Putrescine	0.03	0.16	0.04	0.21
Serotonin	0.60	0.82	0.41	0.60
Spermidine	0.16	0.09	0	0.03
Spermine	0.04	0.06	0	0
Tryptamine	0.77	0.97	0.48	0.74
Tryptophan	0.65	0.72	0.84	0
Tyramine	0.65	0.91	0.53	0.67

in Table III indicates that none of these compounds interferes with histamine in any of the systems. Octopamine in system IV is the only one of the compounds tested which directly interferes with the identification of histamine. Some interference may also come from large amounts of serotonin in systems IV and V, of histidyl-leucine in systems III and IV and of tryptophan in system IV. However, use of more than one system should eliminate any of these interferences. Since large amounts of these substances are needed to cause any interference in the identification of histamine, the likelihood of such occurrences in tuna fish is minimal.

Good separation of histamine from histidine and the other ninhydrin-positive components of the methanolic tuna extracts was achieved with solvent systems III, IV, V and VI, as predicted (see Figs. 1–4). Only in system IV has any interference with histamine identification been encountered with extracts of tuna fish. Such interference manifests itself as tailing of the histamine spot.

Fig. 1. A silica-gel chromatogram developed with solvent system III and sprayed with ninhydrin. Ten-microliter spots of each of the following were applied: No. 1 and No. 7, histamine standard; No. 2 and No. 8, histidine standard; No. 3, methanol extract of sample A; No. 4, methanol extract of sample B; No. 5, 1:10 dilution of methanol extract of sample C; No. 6, methanol extract of sample C. See text for a further description of samples A, B and C.

Fig. 2. A silica-gel chromatogram developed with solvent system IV and sprayed with ninhydrin. The amount and order of samples applied are the same as in Fig. 1.

The development times for systems IV, V and VI were between 50 and 90 min, with additional time necessary for removal of the residual ammonia. With system III, development in the first solvent required 2 h. Since heating to remove the residual butanol was undesirable at this point, the plates were allowed to stand overnight before development with the second solvent, which took an additional 90 min. The long development time for system III may decrease its utility as a rapid screening method.

The minimum detectable amount of histamine observed using this particular ninhydrin spray was 0.4 nmoles (74 ng) with both systems IV and V. Plates run in system III also gave a good response to the ninhydrin spray, although no sensitivity study was done with this system. However, when a plate was developed with system VI, the response to ninhydrin was less definitive. The plates in Figs. 1–4 were spotted exactly the same way, but the histamine spots in Fig. 4 are much less intense than those in Figs. 1–3. This same observation was also made when other visualization reagents were used[24].

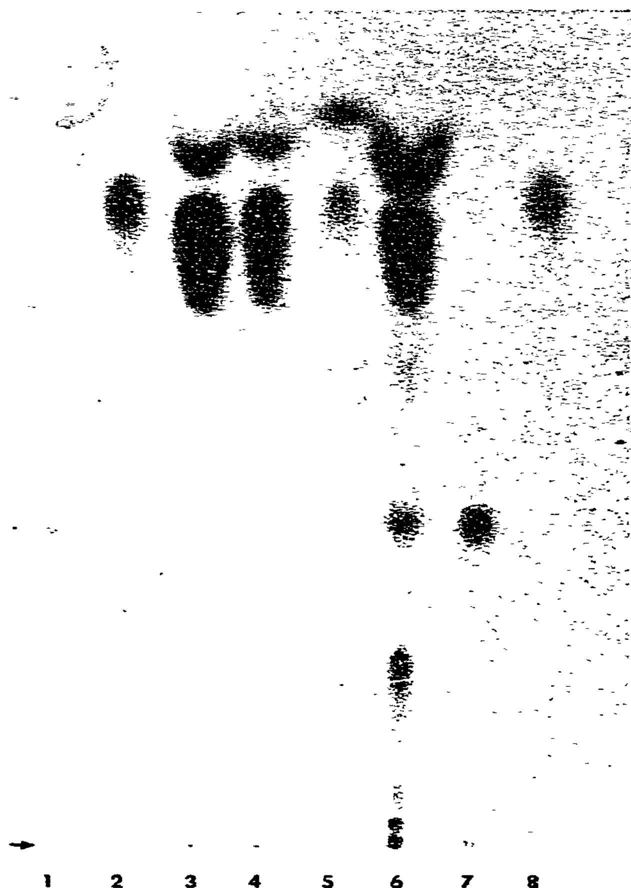

Fig. 3. A silica-gel chromatogram developed with solvent system V and sprayed with ninhydrin. The amount and order of samples applied are the same as in Fig. 1.

As shown in Figs. 1–4, histamine spots are clearly visible with sample B, which had been spiked to a threshold toxicity level of 64 mg histamine/100 g tuna. Since 10 μl of sample B were spotted on each plate, the spots reflect the reaction of 3.5 nmoles (640 ng), which is well above the minimum detectable concentration of histamine. The histamine level in a spot of sample A is 0.6 nmoles (110 ng), which is clearly visible on the plates developed with systems III and IV. The presence of histamine in sample A is just discernible on the plate from system V. These results are tempered by the fact that the histamine in the 1:10 dilution of sample C, the level of which would be 1.6 nmoles/spot, did not react with the ninhydrin as intensely as would have been predicted. The reason for this is unclear.

This result, however, does not minimize the value of these solvent systems in screening tuna samples for potentially toxic levels of histamine. Any one or combination of systems can easily handle a large number of samples routinely. Only those samples found to have histamine levels visually similar to or greater in intensity than a threshold toxicity sample prepared similarly to sample B would have to be subjected

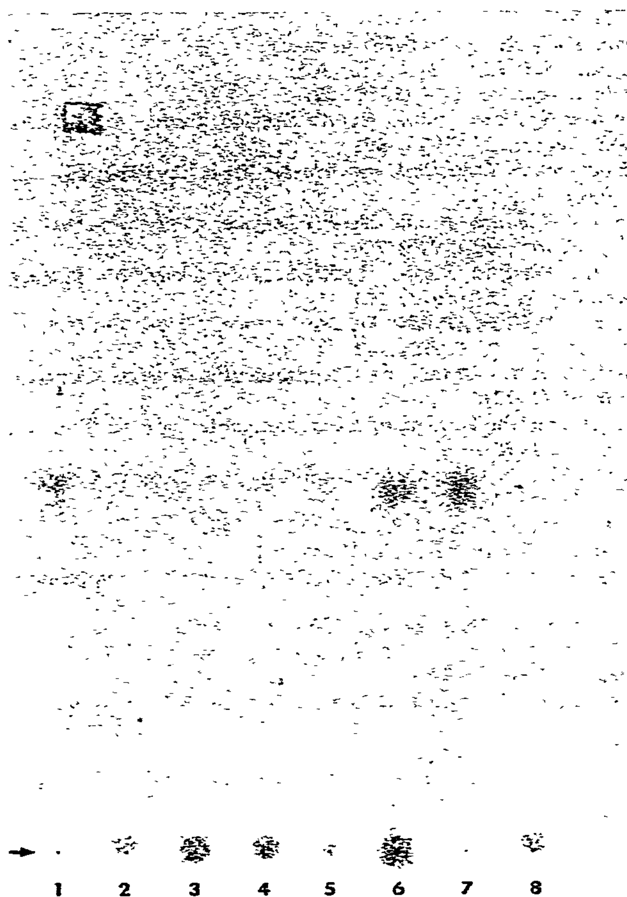

Fig. 4. A silica-gel chromatogram developed with solvent system VI and sprayed with ninhydrin. The amount and order of samples applied are the same as in Fig. 1.

to further quantitative analysis by the fluorometric procedure[16]. Such histamine levels in foods are easily detectable in systems III, IV, V and VI. Of the four methods evaluated, system V presents the most advantages, with system IV being nearly equivalent. Both are rapid, while system III is very time-consuming. In addition, plates developed in both systems IV and V react well with the ninhydrin, while those from system VI are not as sensitive. The only advantage of system V over system IV is that there is no potential for octopamine interference in the identification of histamine. However, the occurrence of octopamine in tuna fish has not been established. A comparative study of fluorogenic visualization reagents more specific for histamine, which may eliminate this objection for system IV, is also in progress[24].

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