Journal of Chromatography, 160 (1978) 227–237

CHROM. 11,107

COMPARISON OF THIN-LAYER CHROMATOGRAPHIC DETECTION METHODS FOR HISTAMINE FROM FOOD EXTRACTS*

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(First received February 17th, 1978; revised manuscript received April 20th, 1978)

SUMMARY

The specificity and sensitivity of four visualization reagents, ninhydrin, *o*-phthalaldehyde (OPT), fluorescamine and *o*-diacetylbenzene (DAB), for the detection of histamine on thin-layer chromatograms developed in several optimal solvent systems were compared. While the ninhydrin reagent is the most sensitive with a detection limit of 0.4 nmoles of histamine, the DAB and fluorescamine reagents offer the most potential as histamine-specific visualization reagents. The OPT reagent was unsatisfactory because the fluorescent spots developed with this reagent were unstable. Of 19 amines tested in addition to histamine, ninhydrin reacted with 17, fluorescamine with 8 and DAB with 15. Since only histidyl–leucine, octopamine, serotonin and tryptophan interfere in the resolution of histamine in any of the solvent systems used, DAB provides some advantages by not reacting with histidyl–leucine. In addition, with the acetone–ammonia (95:5) solvent system, which adequately separates histamine from all other tested amines, no background interference problems were noted when using DAB as the visualization reagent. Background interference problems with the acetone–ammonia system were evident with the other visualization reagents. The sensitivity of the DAB and fluorescamine methods (4 nmoles of histamine) allows both reagents to act as semi-quantitative screening agents for potentially toxic levels of histamine in food extracts. With a variety of food samples, DAB visualization following development in acetone–ammonia (95:5) was the optimal thin-layer chromatographic method.

INTRODUCTION

Considerable interest has been generated in recent years regarding histamine as a possible cause of food poisoning[1–3]. Histamine is often a minor constituent of foods, and these low levels apparently have no toxic effect. However, since histamine

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formation is the result of microbial decarboxylation of histidine, those foods which have been fermented or subjected to bacterial degradation may contain potentially toxic levels of histamine. At the present time histamine concentrations of 100 mg per 100 g food product and above are considered to be toxic[2,4–7].

It has been shown that some scombroid fish products[8–10], as well as cheese[10,11], sauerkraut[12–14] and some fermented sausages[15–17], have relatively high levels of histamine when compared to other foods. A rapid screening procedure is necessary for the routine monitoring of such food products for the presence of an occasionally excessive amount of histamine, which might precipitate an outbreak of foodborne illness. In an earlier study, Lieber and Taylor[18] examined several solvent systems and adsorbents for their potential utility as preliminary thin-layer chromatographic (TLC) screening methods for the detection of histamine in food extracts. Four of the solvent systems evaluated achieved good separation of histamine from the other amine components present in methanolic tuna-fish extracts. Ninhydrin, a general detection reagent for compounds having primary amino groups, was used as the visualization reagent. Although ninhydrin was a satisfactory detection reagent for use with the methanolic tuna-fish extracts, several amines which are found in some of the fermented foods could possibly interfere with the identification of histamine[18] in these foods. Therefore, an investigation of potential histamine-specific detection reagents for TLC was undertaken.

Each of three histamine-specific fluorometric detection reagents, *o*-phthalaldehyde (OPT)[19], *o*-diacetylbenzene (DAB)[20] and fluorescamine[21], was compared to ninhydrin, as well as to each other, with respect to its sensitivity for histamine, its stability and ease of manipulation, its reaction with substances that could interfere with the visualization of histamine by TLC and its utility as a semi-quantitative screening agent. Of these alternative detection reagents, only fluorescamine has been previously adapted as a TLC visualization reagent with any success[22,23]. However, the OPT reagent has been shown to be the most sensitive and specific for detection of histamine in solutions[24]. Development of a relatively sensitive, specific and semi-quantitative TLC method for histamine would allow rapid screening of a wide variety of food products for identification of items that contained potentially toxic levels of histamine. The more tedious, but quantitative, fluorometric method for histamine analysis in foods[10] could then be applied to those items exhibiting high levels of histamine in the TLC screening procedure.

EXPERIMENTAL

Chemicals and supplies

Amino acid, amine and dipeptide standards and visualization chemicals were obtained from the following sources: histamine dihydrochloride, glycine, ninhydrin (reagent grade), OPT and L-tryptophan were purchased from J. T. Baker (Phillipsburgh, N.J., U.S.A.); agmatine sulfate, L-carnosine, cadaverine, L-histidine, 2-mercaptoethanol, 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.); L-histidyl–L-leucine and L-histidyl–L-serine from Vega-Fox Biochemicals (Tucson, Ariz., U.S.A.); DAB from

Polysciences (Warrington, Pa., U.S.A.); and fluorescamine (Roche Diagnostics) from Scientific Products (Menlo Park, Calif., U.S.A.). All other chemicals and solvents used were reagent grade.

Precoated EM silica gel thin-layer plates without fluorescent indicator (EM Labs.) were purchased from VWR Scientific (San Francisco, Calif., U.S.A.). Gelman Chromist laboratory spray units were purchased from Fisher Scientific (Santa Clara, Calif., U.S.A.), and AnaSprā portable laboratory sprayers from Analabs (North Haven, Conn., U.S.A.).

Chunk light tuna, fancy albacore solid white tuna, jack mackerel, sauerkraut, sardines, Italian dry salami, pepperoni, cheddar cheese, frozen comminuted turkey meat and frozen lobster were purchased in local supermarkets. In addition, a sample of canned chunk light tuna in oil, from a recalled lot known to have caused illness, was generously supplied by Dr. Harold Olcott, University of California, Davis.

Methods

General. All amino acid, amine and dipeptide standard solutions used were 2 m*M* in concentration and were prepared as previously described[18]. Methanolic extracts of all food samples were obtained according to the method of Taylor *et al.*[10]. The methanolic extract of one sample of fancy albacore solid white tuna in water was spiked with histamine to a level of 64 mg per 100 g tuna, which approaches the threshold concentration of histamine thought to produce clinical symptoms of toxicity[2,4–7]. In addition, the extract of the tuna sample, containing 300 mg histamine per 100 g tuna, from the recalled lot known to have caused illness was diluted 1:10 with methanol. The histamine content of the recalled lot was determined by the spectrofluorometric method of Taylor *et al.*[10].

The TLC plates were prewashed in acetone and spotted as indicated previously[18]. The following four solvent systems, freshly prepared, were used for the development of the TLC plates: system A, double development with (a) *n*-butanol–acetone–water (2:2:1)[18] and (b) chloroform–methanol–ammonia (12:7:1)[25]; system B, chloroform–methanol–ammonia (2:2:1)[26]; system C, methanol–ammonia (20:1)[27]; and system D, acetone–ammonia (95:5)[28]. The chromatograms were then allowed to air-dry or were gently warmed on a hot plate until the residual ammonia had been removed. Spots were visualized by using one of the reagents listed below.

Ninhydrin spray. This reagent was prepared as previously described[18].

OPT spray. A series of three solutions were sprayed sequentially. The chromatogram was first sprayed with methanolic NaOH until just damp. This solution was prepared by adding 1 ml 10 *N* NaOH to 100 ml methanol. After the plate had air-dried, it was sprayed with a 10-mg/ml solution of OPT in methanol. The final spray was 10% acetic acid, which was applied 1.5 to 2 min after the OPT spray. The plate was viewed immediately under long-wave ultraviolet (UV) light. The OPT solution remained stable for several weeks at room temperature.

Fluorescamine dip. Three solutions were again required. First, the chromatogram was sprayed with methanolic NaOH, as was done with the OPT, and allowed to dry. The plate was then dipped into a solution containing 10 mg fluorescamine in 20 ml acetone and 80 ml hexane. This solution could be stored in the dark for approximately 3–5 days. A flat enamal pan was used for the dipping procedure and was covered with aluminium foil after the plate had been immersed in the liquid.

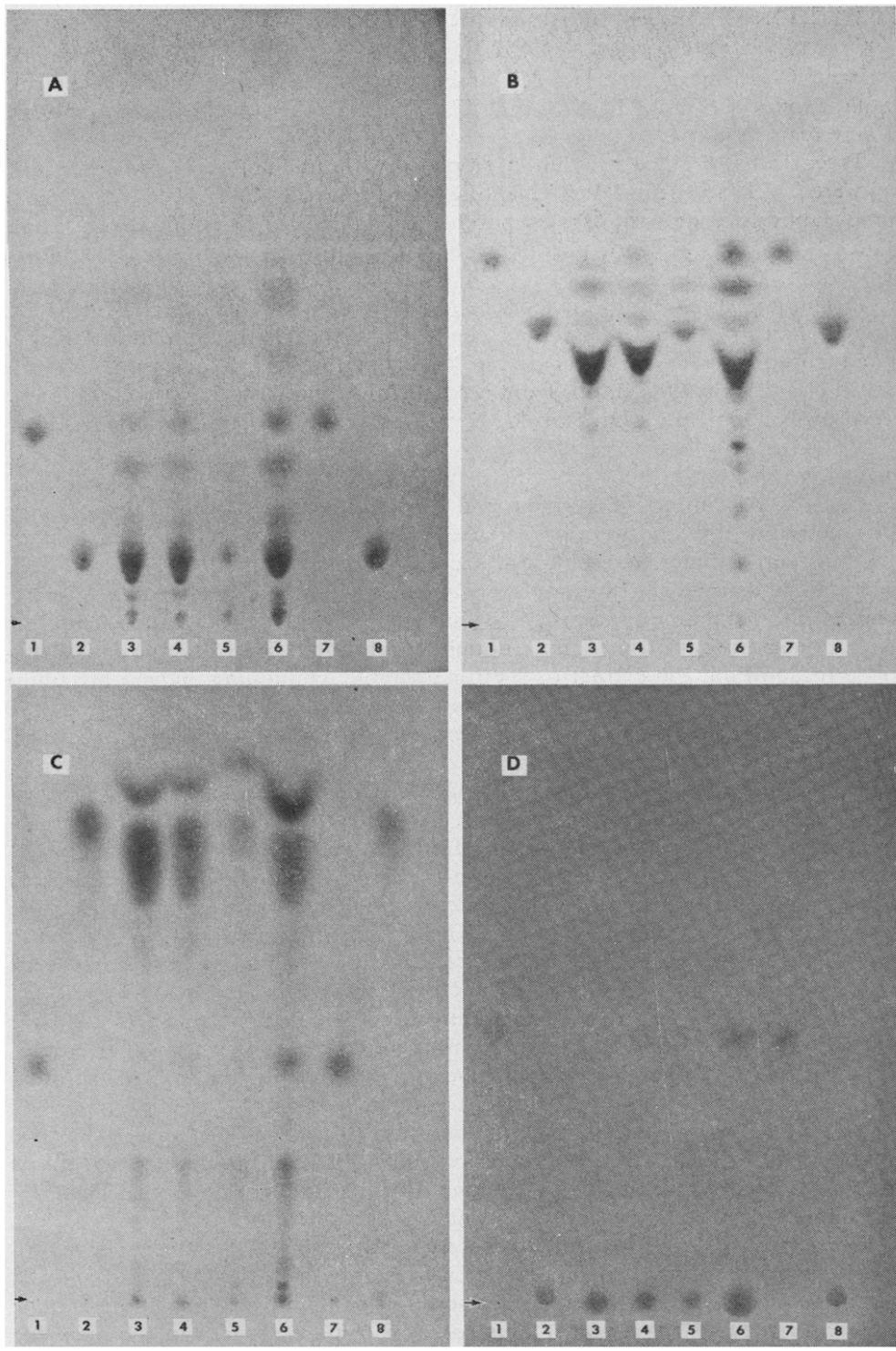

Fig. 1. Comparison of TLC plates visualized with ninhydrin. Plates A, B, C and D were dev with solvent systems A, B, C and D, respectively. Spots of 10 μl of each of the following were a 1 and 7, histamine standard; 2 and 8, histidine standard; 3, methanol extract of chunk ligh 4, methanol extract of albacore solid white tuna spiked with histamine to a level of 64 mg pe tuna; 5, a 1:10 dilution of a methanol extract of a recalled lot of chunk light tuna known t caused illness (30 mg histamine per 100 g tuna); and 6, the undiluted methanolic extract of

The chromatogram remained in contact with the fluorescamine reagent for 15 min. After air-drying, the plate could be viewed under long-wave UV light. At this point, however, all the primary amines present in the applied samples fluoresced. To make this procedure more specific for histamine, the plate was treated a third time by spraying with 0.5 *N* HCl in methanol and heating gently on a hot plate for 30 min. It was then viewed again under long-wave UV.

DAB spray. Three solutions were sprayed in sequential order. First, the layer was made alkaline with methanolic NaOH, as in the OPT and fluorescamine procedures. Then it was sprayed with a solution of 350 mg DAB in 100 ml methanol, and immediately oversprayed with a solution of 500 μl mercaptoethanol in 100 ml methanol. The fluorescent spots formed were made visible under long-wave UV. These spots intensified on standing 15 to 30 min. Both the DAB and mercaptoethanol solutions were stable at room temperature for several weeks.

Sensitivity studies. Histamine sensitivity studies were carried out as described earlier[18]. Plates were developed with both systems B and C and then sprayed with the appropriate visualization reagent.

RESULTS AND DISCUSSION

The results obtained with the ninhydrin spray are shown in Fig. 1. The plates developed with systems A, B and C gave good responses to the ninhydrin. However, when system D was used, the ninhydrin response was much less definitive. All four plates in Fig. 1 were spotted exactly the same way, but the histamine spots on plate D are less intense than those on the other plates. In addition, plates chromatographed in system D tended to scorch easily if they were warmed before spraying with ninhydrin; they also had considerable background reaction after spraying.

The minimum detectable amount of histamine obtained with the ninhydrin spray was 0.4 nmoles (74 ng) with both systems B and C. Chromatograms developed with system A also responded well to this visualization technique, although no sensitivity determination was made with this solvent system or with system D.

The OPT series of sprays gave somewhat more specific results with the tuna extracts (Fig. 2). The histamine and histidine spots fluoresced brightly while the others were less intense or absent when compared to the ninhydrin results. The histamine in the spiked tuna extract (64 mg per 100 g), as well as in the undiluted extract of the recalled tuna (300 mg per 100 g), was visible in these chromatograms, while that in the regular tuna (11 mg per 100 g) and in the 1:10 dilution of the recalled lot (30 mg per 100 g) was not. Thus, this OPT spray could act as a semi-quantitative screening agent. However, the reaction of OPT on the TLC plate was unstable. The fluorescence of the spots began to diminish if the plate was left in room light for even a short period of time, and it diminished more rapidly upon exposure to UV light. Thus the plates had to be examined immediately after spraying with the acetic acid reagent. For this reason no sensitivity determinations were made for the OPT reagent. All the TLC plates developed in system D had high background interference and could not be photographed satisfactorily.

The fluorescamine dip also gave fluorescent spots, which were much more stable than those formed by the OPT. The acid–heat treatment rendered this visualization technique highly specific for histamine. Fig. 3 shows the plate before and after the

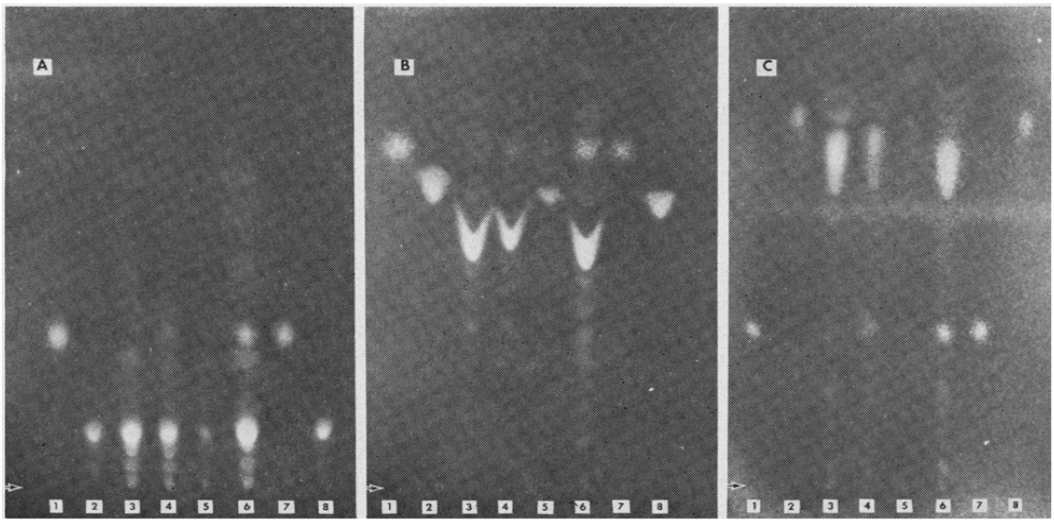

Fig. 2. Comparison of TLC plates visualized with OPT. The amount and order of samples applied, as well as the three developing solvents used, are the same as in Fig. 1. Because of high background interference, the plate developed with solvent D could not be photographed.

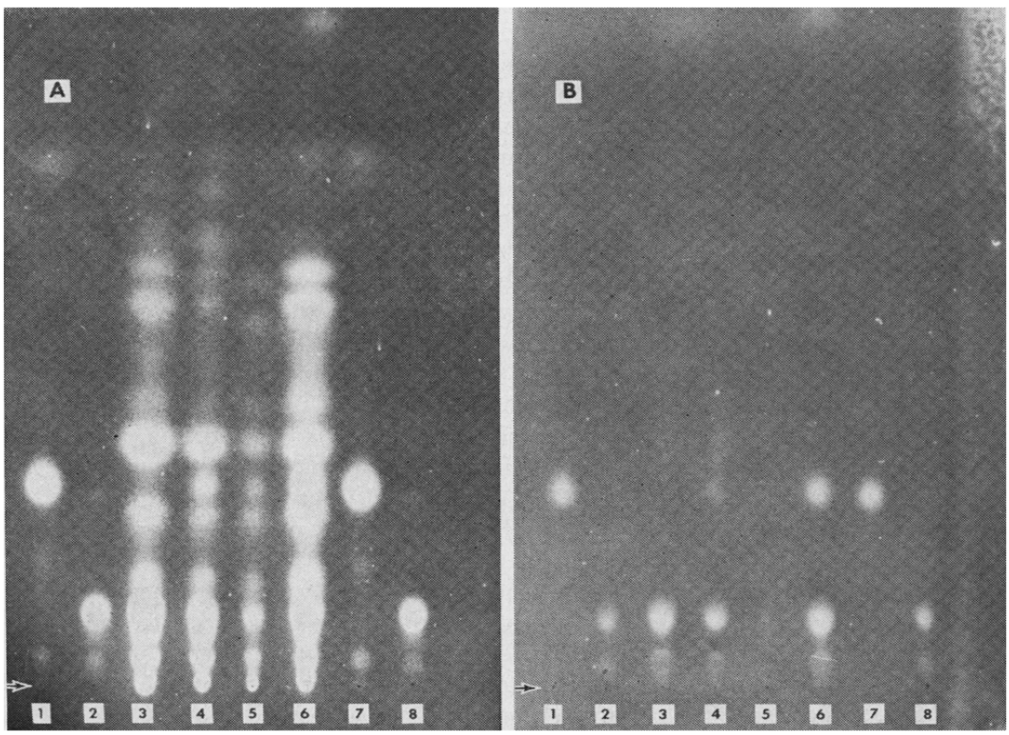

Fig. 3. Comparison of TLC plates developed in system A and visualized with fluorescamine before acid–heat treatment (plate A) and after (plate B). The amount and order of samples applied are the same as in Fig. 1.

acid–heat treatment following development with system A and visualization with fluorescamine. Examples of plates which have been run in the four solvent systems and have undergone the acid–heat treatment of the fluorescamine procedure can be seen in Fig. 4.

The DAB visualization procedure (Fig. 5) seems to be less reactive with other compounds migrating near histamine than the ninhydrin spray, especially when system B has been used for the separation. As a result, it appears to be more histamine-specific than ninhydrin. In addition, the spots formed with DAB are more pronounced than those obtained after the acid–heat treatment of fluorescamine.

The minimum detectable quantity of histamine after treatment with DAB or fluorescamine with acid–heat was approximately 4 nmoles when chromatographed in systems B and C. This represents a 10-fold loss in sensitivity when compared to ninhydrin. However, this need not be construed as a disadvantage. Use of a screening method implies that only those samples meeting a certain criterion are to be separated from a host of others which do not. The minimum detectable quantities for both DAB and fluorescamine approximate the histamine level present in the spiked tuna extract. Therefore, any sample containing potentially toxic levels of histamine could be easily detected. Only those samples found to have histamine levels visually similar to or greater in intensity than a prepared threshold toxicity sample such as the spiked tuna extract would have to be subjected to further, quantitative analysis. With both the DAB and fluorescamine treatments, the histamine present in the spiked tuna extract is easily detectable, while the histamine in the extracts of regular tuna and the 1:10 dilution of the recalled lot are barely visible (Figs. 4 and 5). This observation is apparent with the chromatograms developed in all four systems and treated with DAB, and those developed in all but system D and treated with fluorescamine.

A comparison of the reactions of these four visualization techniques with biogenic amines other than histamine is given in Table I. TLC of these amines in systems A, B, C and D has shown that only histidyl–leucine, octopamine, serotonin and tryptophan pose any interference with the migration of histamine[18]. Even though ninhydrin gave a positive reaction with all but two of the amines tested, it still worked satisfactorily for the visualization of tuna-fish extracts since none of the potentially interfering compounds have been identified as being present in the fish. However, these four amines may be present in other food products[29,30], and their presence could limit the utility of ninhydrin visualization for other foods. Any discussion of the specificity of the OPT spray with any of these amines is futile because of its instability as a spray reagent. For this reason all of the amines were not tested for their response to OPT on the TLC plates. Although fluorescamine forms fluorescent spots with the least number of the compounds tested, all four of the amines determined to be potential interferences in the determination of histamine by TLC react with the fluorescamine dip, and their spots remain fluorescent after the acid–heat treatment. However, it was found that histidyl–leucine does not react with the DAB spray. Visualization by this method, then, leaves only three potentially interfering substances in the separation and identification of histamine by TLC. Since none of these three amines has an R_F value similar to that of histamine in system D[18], use of the DAB spray in conjunction with system D will eliminate any of these interferences. In addition, use of two of the three remaining systems with either the DAB spray or the fluorescamine dip will also eliminate any interference by these compounds.

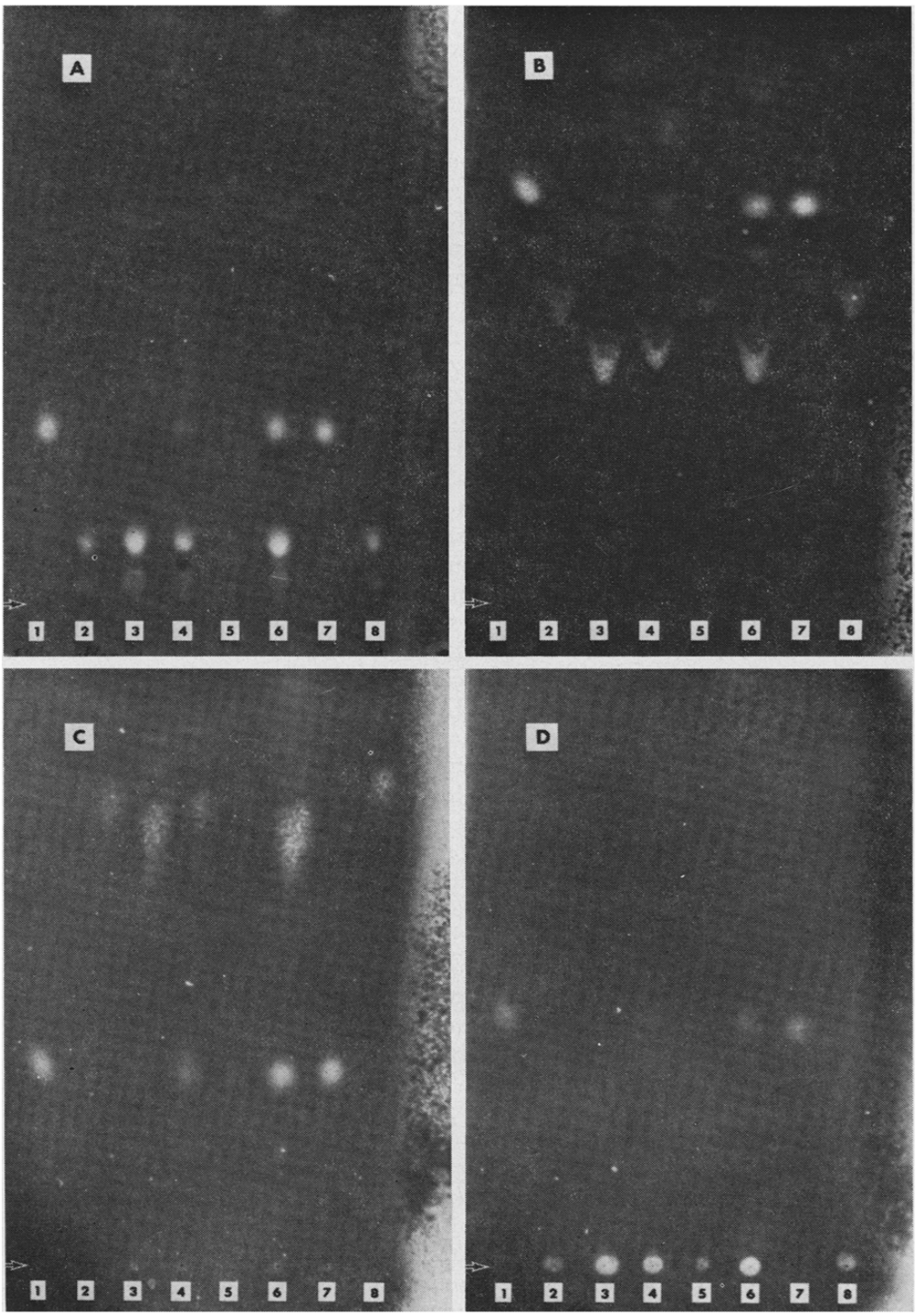

Fig. 4. Comparison of TLC plates visualized with fluorescamine, sprayed with strong acid and heated. The amount and order of samples applied, as well as the developing solvents used, are the same as in Fig. 1.

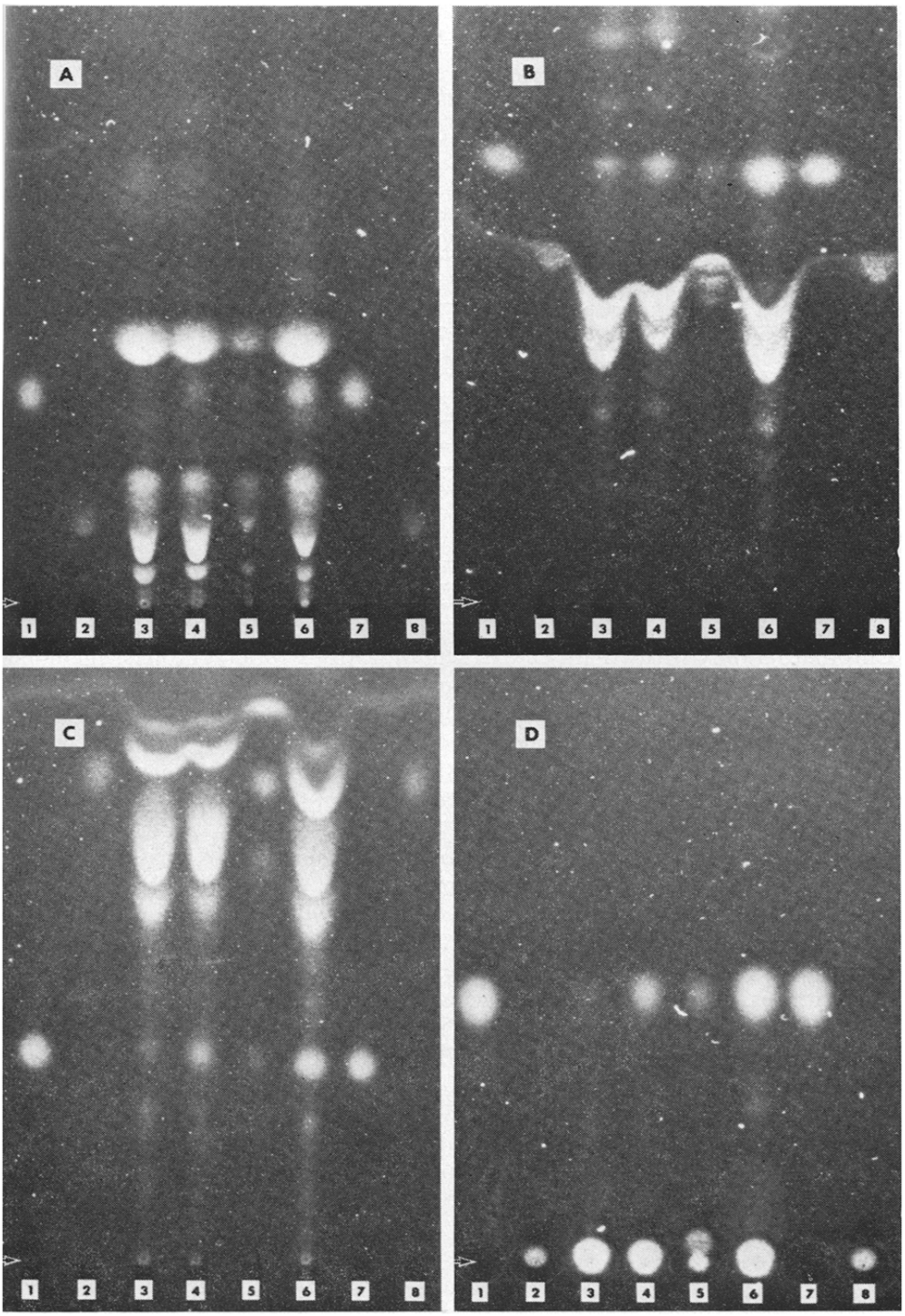

Fig. 5. Comparison of TLC plates visualized with DAB. The amount and order of samples applied, as well as the developing solvents used, are the same as in Fig. 1.

TABLE I
REACTIONS OF THE VISUALIZATION METHODS DESCRIBED WITH VARIOUS BIOGENIC AMINES

Amine	*Ninhydrin*	*OPT*	*Fluorescamine*	*DAB*
Histamine	+	+	+	+
Histidine	+	+	+	±
Histidyl–leucine	+	+	±	−
Octopamine	+	±	±	+
Serotonin	+	+	+	+
Tryptophan	+	+	+	+
Lysine	+		−	+
Glycine	+		−	+
Carnosine	+		−	+
Histidyl–serine	+		±	−
Cadaverine	+		−	+
Agmatine	+		−	+
β-Phenylethylamine	+		−	+
Putrescine	+		−	+
Spermidine	+		−	+
Spermine	+		−	+
Thiamine	−		−	−
Trimethylamine	−		−	−
Tyramine	+		±	+
Tryptamine	+		+	+

Chromatography of methanolic extracts of various food products, including luncheon meats, salami, sauerkraut, lobster, mackerel, sardines and cheddar cheeses, has been as successful as that of the tuna extracts. The ninhydrin and DAB visualization methods for these extracts were compared. All but two of the extracts examined appeared to have histamine levels well below that of the spiked tuna sample. Except in these two instances, histamine was detected on the ninhydrin-sprayed plates, but not on those sprayed with DAB.

From the results presented here and in a previous paper[18] it is evident that TLC provides a rapid means for the determination of potentially toxic levels of histamine in foods. The four solvent systems of choice will provide good separation of histamine from other amines in tuna-fish extracts and those of other food products. A combination of two or more of these systems will separate histamine from other amine interferences. System D effectively separates histamine from all other amines tested thus far. The DAB spray procedure has advantages over the fluorescamine dip and the ninhydrin spray in that the DAB reacts well with the plates developed in system D, has well defined spots and is somewhat more specific. However, both the DAB and fluorescamine methods provide sufficient sensitivity to give a semi-quantitative indication of samples which must undergo a more rigorous analysis for histamine. Use of these procedures will simplify and shorten the analysis time for histamine determinations in the food processors' quality control laboratories, as well as in regulatory laboratories.

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