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Note

Optimal use of fluorescamine for *in situ* thin-layer chromatographic quantitation of amino acids

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Fluorescamine (Fluram®; Roche) has become widely used as a reagent for primary amines[1]. Udenfriend *et al.*[2] showed that the reaction proceeds well at room temperature in aqueous solutions. This reagent has been shown to react selectively with primary amines, amino acids, peptides, and proteins to form fluorescent products and has been applied to automated analysis of effluents of ion-exchange columns by these authors. A solution of fluorescamine in acetone has been used in a spray for amino acids on thin-layer chromatograms by Felix and Jimenez[3], who reported the use of a triethylamine spray to stabilize the fluorescent product on the thin layer. Sherma and Touchstone[4] utilized the reaction for spectrofluorometry *in situ* of amino acids separated on thin-layer chromatograms.

Most of the studies concerning fluorescamine have been with the amine in solution. Few studies are available which assess the parameters for optimal quantitation of amino acids on thin-layer chromatograms using this reagent. The present report shows the importance of time and manner of spraying on the intensity of fluorescence of various amino acids separated by thin-layer chromatography (TLC).

EXPERIMENTAL

Materials and equipment

Commercial, reagent-grade amino acids were chosen for study, representing four acid types, *viz.* alanine and glycine (aliphatic hydrocarbons), aspartic acid (acidic), phenylalanine (aromatic), and tyrosine (phenolic). A series of standard solutions was prepared for each acid by appropriate dilution of 1 μg/μl stock solutions. The acids were dissolved in water, with the exception of tyrosine, which was dissolved in a dilute NaOH solution.

Fluram (fluorescamine) (4-phenylspiro(furan-2(3H),1′-phthalan)-3,3′-dione), supplied by Roche Diagnostics, Division of Hoffman-LaRoche (Nutley, N.J., U.S.A.), was used in solution with acetone (25 mg/100 ml). Dimethylsulfoxide or dimethylformamide solutions were of the same concentration.

Solvents were reagent grade, redistilled in glass. Adsorbosil-5–silica gel precoated thin layers, 250 μm on 20 × 20 cm glass plates (Applied Science Labs., State

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College, Pa., U.S.A.) were used as received after scoring into twenty parallel lanes with a Schoeffel scoring device.

A Schoeffel SD 3000 spectrodensitometer equipped with an SDC 300 density computer was used for *in situ* scanning of the layers. The reflectance mode with incident light at 365 nm was used. Emission was measured with a 400-nm cutoff filter.

Method

A series of samples (2.5–10 μl) representing amounts up to 1 μg of each acid were spotted or streaked in alternate lanes, 2 cm up from the bottom of the layer, using a Hamilton 10-μl syringe. After drying, the plate was developed in a solvent-saturated, paper-lined rectangular TLC tank to within 1 cm of the top of the plate. Developing solvents used were *n*-butanol–ethyl acetate–glacial acetic acid–water (1:1:1:1) and *n*-butanol–acetic acid–water (4:1:1). After development, the layers were dried in a hood at room temperature using a cold air blast from a hair dryer. The method of Felix and Jimenez[3] used heating at 110° for 10 min.

The following investigations were made: (1) A comparison was made of heating at 110° for 10 min *versus* using the hair dryer with ambient air for 30 min using spray method (a) below. (2) A comparison among the following spraying methods was made: (a) Spray with triethylamine in methylene chloride–Fluram in acetone–triethylamine in methylene chloride (original method[3]). (b) Effect of time on development of fluorescence. (c) Spray only with Fluram in dimethylsulfoxide. (d) Spray only with Fluram in dimethylformamide.

RESULTS AND DISCUSSION

Table I shows that heating at 110° for 10 min to dry the chromatograms prior to spraying resulted in poor results with some amino acids. A larger fluorescence intensity was obtained when the plates were dried only at room temperature for 30

TABLE I

EFFECT OF PLATE DRYING ON INTENSITY OF FLUORESCENCE

Developing solvent: *n*-butanol–ethyl acetate–glacial acetic acid–water (1:1:1:1). The chromatograms were scanned *in situ* 15 min and 3 h after spraying.

Amino acid	*Peak area of fluorescent peak from TLC scan (mm²)*			
	Heat, 10 min, 110°		*Hair dryer, 30 min, room temperature*	
	15 min	*3 h*	*15 min*	*3 h*
Alanine	390	445	400	525
Aspartic acid	188	230	288	396
Tyrosine	400	376	564	600
Phenylalanine	302	282	473	528
Arginine	360	410	312	378
Histidine	134	254	168	270
Serine	305	408	721	760
Cysteine	450	460	404	875

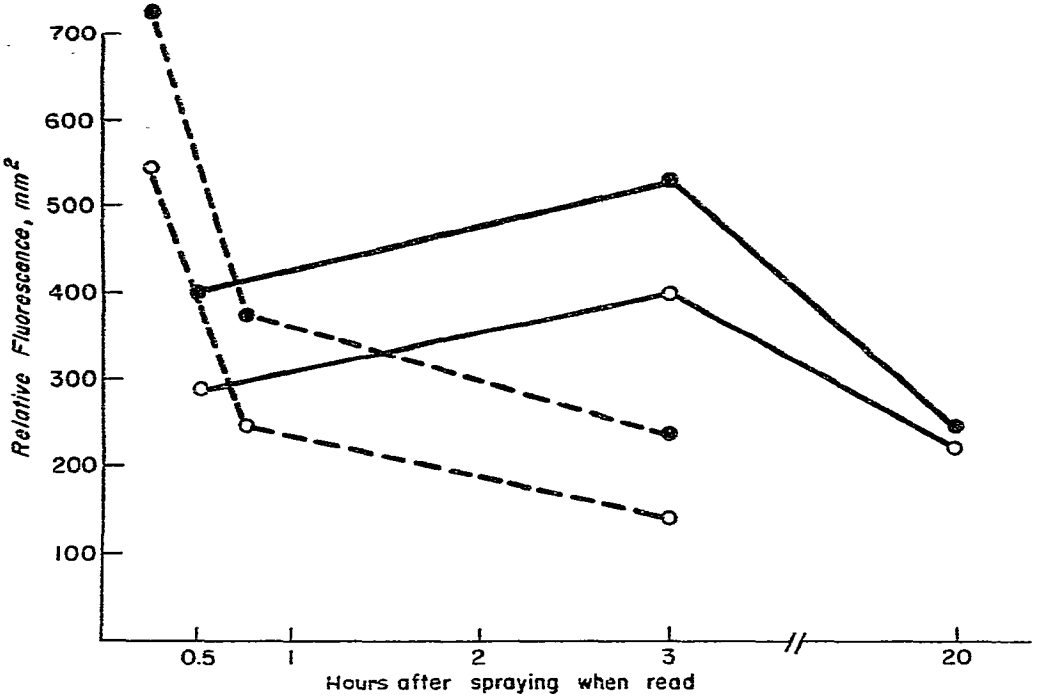

Fig. 1. Effect of spraying sequence on intensity of fluorescence obtained with Fluram and amino acids on TLC. ●, Alanine; ○, aspartic acid; ———, pre and after spray; – – –, no spray.

min using an ambient air current from a hair dryer. This result was obtained when any of the solvents above was used for development of the chromatograms.

Absorbosil-5, as reported in the previous paper[4], provided the background on scanning at 365 nm that was the most free of background interference.

Using the improved drying method and the plates of choice, a comparison among the spraying methods was performed. Spraying in a single operation, rather than the three-step original spraying method, would be less fraught with problems of reproducibility and would be easier as well as time-saving.

Spraying with solutions of Fluram in dimethylsulfoxide or dimethylformamide proved to be suitable in that no triethylamine stabilization was required. Any one of the three spraying methods gave a fluorescent reaction for each amino acid.

The reaction was time dependent, regardless of the spraying method used. The emission appeared to develop a high intensity within 30 min, followed by a rapid decline. Then intensity increased to a maximum at 3 h, to be followed by a second but less rapid decline. Different amino acids reacted at different rates at the beginning. Fig. 1 shows that the prespraying with the triethylamine solution, before and after spraying with the Fluram, stabilized the fluorescence when acetone was used as solvent. The figure also shows how the emission intensity varied with the time after spraying at which the layers were scanned. These results point out the importance of assessing the time factors in quantitation of amines using the Fluram reagent. Table II gives a comparison of the fluorescence intensities of a number of amino acids

TABLE II

FLUORESCENCE INTENSITY OF AMINO ACIDS USING DIFFERENT FLURAM SOLUTIONS

Developing solvent: *n*-butanol–acetic acid–water (4:1:1). The chromatograms were scanned *in situ* 30 min and 3 h after spraying.

Amino acid	*Area under peak of emission scan (mm²)*					
	Solution of Fluram in					
	Acetone		*Dimethyl sulfoxide*		*Dimethylformamide*	
	30 min	*3 h*	*30 min*	*3 h*	*30 min*	*3 h*
Tyrosine	770	252	512	1012	274	480
Serine	1012	398	385	870	252	464
Phenylalanine	295	124	480	1010	615	850
Histidine	79	41	78	214	70	188
Cystine	825	455	568	1260	380	700
Arginine	1402	590	162	680	735	370

visualized after spraying with Fluram in dimethylformamide or dimethylsulfoxide When using these sprays, no triethylamine spraying was required.

These results indicate that either dimethylformamide or dimethylsulfoxide can be used as a vehicle for the Fluram reaction without the use of the stabilizing triethylamine. Apparently, these solvents provide the conditions necessary for the reaction between the Fluram and the amino grouping. The solvents do have higher boiling points than acetone, and could have longer contact times than when acetone was used for the solvent. Regardless of the spraying method used, if quantitation is to be done, calibration curves and reaction conditions must be standardized if reproducibility is to be achieved. Furthermore, for each amino acid, calibration curves must be obtained.

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