CHROM. 3719

Improved thin-layer chromatographic separation of amanita toxins on Silica Gel G chromatoplates*

Amanita toxins are polypeptides that contain unusual amino acids[1–3]. These toxins are present in certain species of mushrooms and thus are of interest to physiologists, pharmacologists, and biochemists. Because they are large complex molecules, study of their structures can provide knowledge ultimately useful in the stereochemistry of biological molecules. The chemistry and toxicology of amanitins have been studied by WIELAND[2,3]. A thin-layer chromatographic method for the separation and detection of α- and β-amanitins in mushroom extracts is described by SULLIVAN, BRADY AND TYLER[4]. A methanolic extract of the mushroom is chromatographed on a Silica Gel G chromatoplate, which is then developed with methanol–methyl ethyl ketone. The positions of the amanitins are detected by spraying the plate with a solution of *trans*-cinnamaldehyde in methanol, drying the sprayed plate, and exposing it to the vapors from hydrochloric acid. Discrete lavender-purple areas indicate the positions of the amanitins. The application of the method to the isolation and detection of the toxins from mushrooms is described in further works[5,6].

A project was undertaken by C. K. JOHNSON (Chemistry Division, ORNL) and E. F. PHARES (Biology Division, ORNL) to prepare significant quantities of the amanitin toxins by batch fermentation growth of the mycelium of the mushroom *Galerina marginata* according to the method of BENEDICT, TYLER, BRADY AND WEBER[5]. The thin-layer chromatographic method of SULLIVAN, BRADY AND TYLER[4] was used to follow the progress of the production. During this work, some improvement in the chromatographic separation was realized by means of shaped chromatoplates and, for some samples, also overrun of the development.

Experimental

Apparatus. The Desaga/Brinkmann Minimum Recommended Assembly for TLC (Brinkmann Instruments Inc., Westbury, N.Y.) was used to prepare Silica Gel G chromatoplates of 200-μ nominal layer thickness. The chromatoplates were activated at 105° for 90 min, as recommended[4], and were stored at 50° (ref. 7); Boekel model No. 1078 ovens were used. The activated chromatoplates were shaped by means of a polyethylene nozzle of ~ 1-mm-width tip, which was attached to a vacuum line, and a No. 6 cork borer. Lang-Levy micropipets were used to deposit the extracts on the chromatoplates. The developing tank was the Desaga type. Developed chromatoplates were exposed to vapors of concentrated hydrochloric acid in a 7 1/8 in. wide by 9 3/8 in. long by 12 3/4 in. deep chromatographic jar provided with a plate-glass cover; the chromatoplates were set edgewise on a crystallizing dish, in the jar, that contained concentrated hydrochloric acid.

Reagents. The reference source of amanitins was a portion of a standard sample of *Amanita phalloides* powder provided by Professor R.G. BENEDICT, College of Pharmacy, University of Washington. Concentrated methanolic extracts of the

* Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

Amanita phalloides were obtained by Soxhlet extraction and flash evaporation of the Soxhlet extract.

Silica Gel G, "according to Stahl" (E. Merck & Co.), was purchased from Brinkmann Instruments Inc.

trans-Cinnamaldehyde was Reagent 77 from Distillation Products Industries, Rochester, N.Y.

All other reagents were analytical-reagent grade.

Procedures. The general procedures used were those described by SULLIVAN, BRADY AND TYLER[4]. The exact details of the procedures are recorded as a method[8].

Overrun of the development was achieved by removing a narrow band (~ 1 mm wide) of the Silica Gel G from along a line located at the desired maximum distance of solvent travel and then allowing the chromatoplate to remain in the developing solvent for some time (usually 10 min) after the solvent front had reached that line.

Results and discussion

The chromatograms compared in the color prints (Fig. 1) indicate the better resolution of the amanitins and the greater band sharpness that are achieved by use of shaped layers of adsorbent; the distance of travel of the developer was 10 cm from the origin.

The sensitivity of the method is difficult to define. It is reported that as little as 0.3 μg of pure α- or β-amanitin can be detected with certainty by this procedure[6]. However, limitations with respect to the total amount of an amanitin and the total volume of the test solution spotted on the chromatoplate may vary. Test-portion volumes of 2 μl, spotted in replicate one on top of the other with drying of each spot before deposition of the next, have been satisfactory with 200-μ-thick layers of Silica Gel G. The success of the separation and the effectiveness of the detection of the amanitins depend somewhat on the condition of the Silica Gel G layer and the freshness of the developer solvents. It has been recommended that chromatoplates older than three days should not be used[7]. Some chromatoplates older than this were used satisfactorily, but they seemed to be less sensitive than freshly prepared chromatoplates and to show a faint-violet background color when sprayed with *trans*-cinnamaldehyde reagent. Commercially available Silica Gel G plates did not give satisfactory results. Methyl ethyl ketone used from the one-quarter that remained in a 2-l bottle opened sometime before gave a pronounced violet background color. For maximum sensitivity, the time between the spotting and the development of a plate should be kept as short as possible; chamber saturation during development is recommended.

In the formation on the chromatoplate of the lavender-purple complexes of the amanitins with *trans*-cinnamaldehyde, it is important that the chromatoplate be surrounded by strong vapors from concentrated hydrochloric acid. The lavender-purple color begins to appear about 5 min after a plate has been exposed to the vapors; maximum color intensity may not be reached until 20 to 30 min later. Because the color fades as soon as a plate is removed from the vapors, the chromatograms must be documented almost immediately. Photocopying the developed chromatoplates by the Xerox method is a quick way to document them.

The R_F values for the amanitins vary with the shape, degree of activation, thickness, and age of the Silica Gel G chromatoplates. Also, the amount of extraneous material in the test portion affects the R_F values. The magnitude of the R_F values of

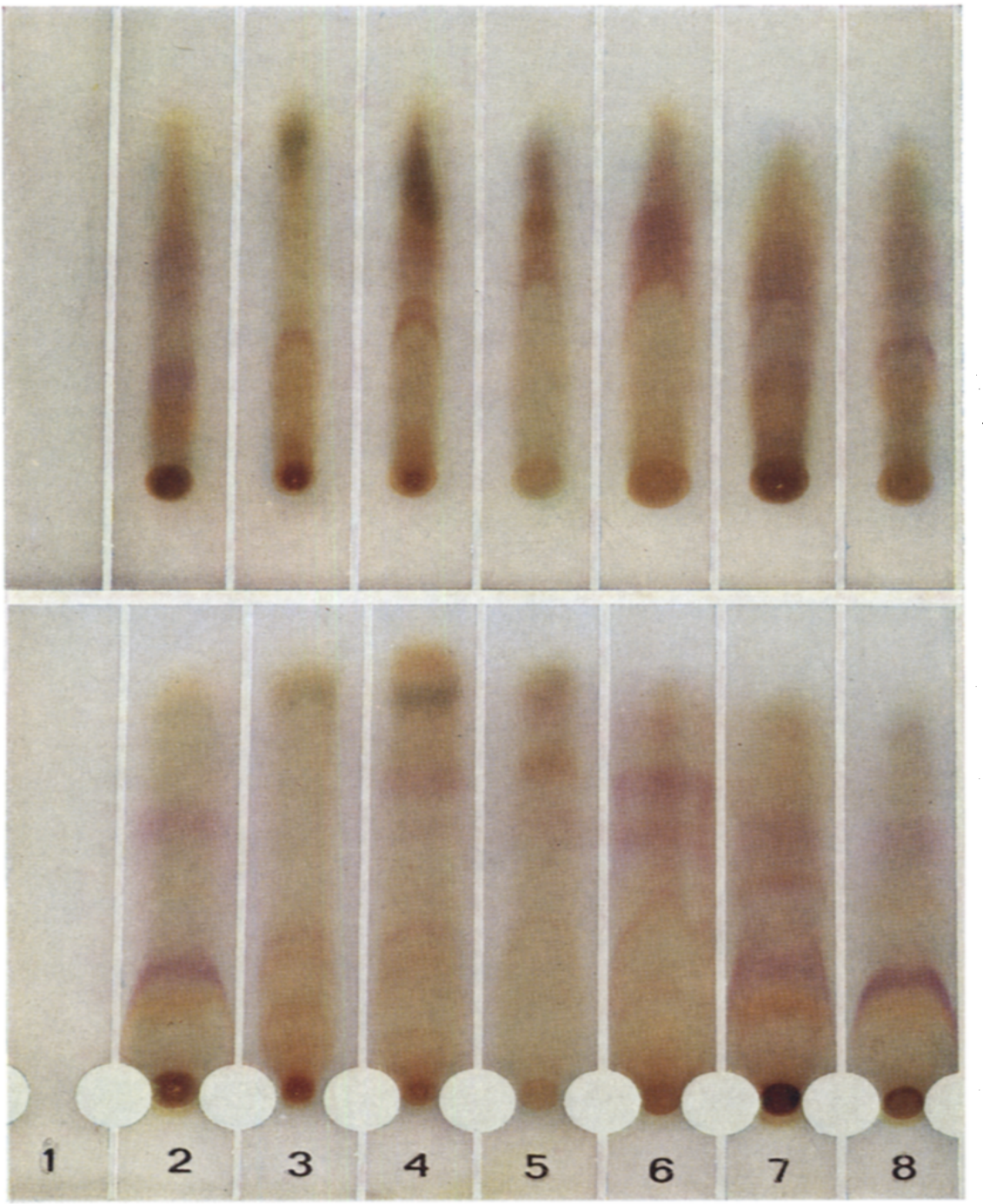

Fig. 1. Photographs of chromatograms of amanitins that show the improved separation achieved with shaped chromatoplates. 1 = Reagent blank; 2 = reference *Amanita phalloides* (lipid-free old extract); 3 = *Galerina marginata* (after 7-day fermentation); 4 = *Galerina marginata* (after 18-day fermentation); 5 = *Galerina marginata* (composite from low-level samples); 6 = *Galerina marginata* (composite from higher-level samples); 7 = reference *Amanita phalloides* (lipid-containing fresh extract); 8 = reference *Amanita phalloides* (lipid-free fresh extract).

the three types of amanitins is in the order: $\beta < \alpha < \gamma$; R_F values for the amanitins on Silica Gel G are given in three publications[4-6].

By this method, some 130 methanolic extracts from *Galerina marginata* and *Amanita phalloides* were analyzed satisfactorily. Both lipid-containing and lipid-free extracts were among them. The samples were derived from pellets harvested during evaluations of fermentation equipment and of anti-foam agents and during studies of the effects of antibiotics on bacteria-contaminated fermentation batches. The results were used to establish suitable fermentation conditions and optimum pellet-harvest times, to measure the effectiveness of the fermentation runs, and thus to guide the course of the preparation of the amanita toxins.

Acknowledgements

Information conveyed by personal communications to C. K. JOHNSON from Professors R. G. BENEDICT and L. R. BRADY of the College of Pharmacy, University of Washington, Seattle, Washington, made this work possible. H. L. HOLSOPPLE of the ORNL Analytical Chemistry Division prepared the methanolic extracts. The invaluable assistance of these persons is gratefully acknowledged.

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Received July 31st, 1968