*Journal of Chromatography* 116 (1976) 155–161


CHROM. 8645

# BIOAUTOGRAPHIC DETECTION OF MYCOTOXINS ON THIN-LAYER CHROMATOGRAMS

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(Received July 18th, 1975)

## SUMMARY

A method for the bioautographic detection of mycotoxins on thin-layer chromatograms by using *Artemia salina* larvae is described. The method was tested on standard samples of mycotoxins (aflatoxin $B_1$, kojic acid and sterigmatocystin) and on the extracts from toxicogenic fungi isolated from different sources.

## INTRODUCTION

Bioautography is used in the chromatography of biologically active substances, especially antibiotics. A recent critical review of the application of bioautography as a special detection method in the paper and thin-layer chromatography of antibiotics[1] indicated the wide range of biological systems used for bioautography: animal viruses, bacteriophages, bacteria, fungi, protozoa, algae and animal cells.

In the study of the toxicity of mycotoxins, *Artemia salina* larvae[2] have been shown to be a suitable model organism. A method for the bioautographic detection of mycotoxins on chromatograms has been developed that is suitable for the initial stages of the screening of mycotoxins when they are available only in crude extracts, either from pure cultures of fungi or contaminated food, fodder and raw material for foodstuffs as well as in instances when physical and especially chemical detection cannot be applied to chromatograms. The bioautographic detection is followed in our study of mycotoxins by a systematic analysis by thin-layer chromatography[3], which, together with other methods, helps to identify the known mycotoxins and to detect unknown mycotoxins.

## MATERIALS AND METHODS

### *Test organism*

*Artemia salina* larvae are grown from commercially available eggs in Frank's medium[4]. To 750 ml of tap water (total hardness of which is 8–14°, German scale) two teaspoonfuls (*ca.* 12.0 g) of sodium chloride (non-iodinated) are added and the pH of the medium is adjusted to 7.4 with 1 *N* potassium hydroxide solution. One

teaspoonful (*ca.* 0.2 g) of eggs of *A. salina* is added to the medium, which is incubated at 28° while subjecting it to intensive aeration. Larvae appear within 18–24 h. The medium is made up daily to the original volume with distilled water.

*Mycotoxins*

We used aflatoxin $B_1$ (Dr. L. Shoetwell, U.S. Department of Agriculture, Northern Research Laboratories, Peoria, Ill., U.S.A.), kojic acid (this Department) and sterigmatocystin (Dr. P. S. Steyn, National Chemistry Research Laboratories, Pretoria, South Africa). Standard solutions of mycotoxins were prepared at a concentration of 1 mg/ml in the following solvents: benzene–acetonitrile (98:2) for aflatoxin $B_1$, ethyl acetate for kojic acid and chloroform for sterigmatocystin.

*Solvent systems*

Systems A, B, C, D, E, F, G and H from ref. 3 (this issue, p. 143) were used.

*Chromatography of the standards of mycotoxins*

Pre-coated thin layers on Silufol sheets (Kavalier, Votice, Czechoslovakia) (20 × 20 cm) were used; they were cleaned prior to chromatography by development in the system in which the sheets were subsequently developed with samples. From our set of systems for the systematic analysis of mycotoxins[3] the following were used: B, toluene–ethyl acetate–90% formic acid (6:3:1); and D, chloroform–methanol (4:1).

On a developed sheet, 10 $\mu$l of standard solutions of mycotoxins (≡ 10 $\mu$g of substance) were applied and developed in system D to a height of 10 cm. Simultaneously, Silufol sheets without standards were developed in systems B and D.

*Bioautographic detection of mycotoxins on chromatograms*

From the developed chromatograms on Silufol sheets, 1.5 × 10-cm strips were cut out vertically, and identified as follows:

strip a: a sheet developed in system D;

strip b: a sheet developed in system D (cleaning) and with a sample of sterigmatocystin developed again in this system;

strip c: a sheet cleaned in system D and with a sample of aflatoxin developed again in system D;

strip d: a sheet cleaned in system D and with a sample of kojic acid developed again in system D;

strip e: a sheet developed twice in system D (without sample);

strip f: a sheet developed once in system B (without sample).

These strips were cut into 1.5 × 1.5-cm squares and adsorbent from the squares was scraped into separate test-tubes. Into each test-tube, 1 ml of the suspension of *Artemia salina* larvae was added and thoroughly stirred, and from each tube 0.2-ml portions were pipetted into four porcelain wells. On average, 20–40 larvae were present in each well. The wells were checked at zero time with an SM XX stereoscopic microscope (Zeiss, Jena, G.D.R.) at a magnification 1.6 × 6.3 for the presence of dead larvae. The wells were covered with micro-scale cover slips in order to prevent evaporation of the solutions. Incubation was carried out at 20–22° and

after 16 h the dead larvae in each well were counted under the stereoscopic microscope. The remaining living larvae were then killed by adding a few drops of chloroform and all larvae were counted again. On the white background of the well, orange larvae were clearly visible and could be distinctly discerned from brown ball-like eggs. The percentage mortality for all samples was calculated[2].

*Cultivation of fungi*

The use of the bioautographic detection of unknown mycotoxins in thin-layer chromatography was tested on crude extracts of secondary metabolites from 33 fungi isolated from various foodstuffs. From well sporulated cultures on slant agars (PCA medium: 200 g of potatoes, 100 g of carrots, 20 g of agar and 1000 ml of water), a spore suspension was prepared. Roux flasks of volume 2 l, containing 200 ml of YES medium[5], were inoculated with this suspension and cultivated under static conditions at 28° for 7–21 days.

*Extraction*

Mycelium of the grown culture and medium were separated by filtration. Mycelium was extracted twice with 50 ml of chloroform, filtered and the combined chloroform extracts were dried over anhydrous calcium chloride and evaporated to dryness under reduced pressure at 50°. The residues were dissolved in 2-ml volumes of chloroform. From the filtrate of the medium, 85 ml were taken and the pH was adjusted to 3.4 with dilute sulphuric acid (1:10). The filtrate was extracted with 50 ml of chloroform by shaking in a separating funnel and the aqueous layer was extracted again with 35 ml of chloroform in a separating funnel. The combined chloroform extracts were dried over anhydrous calcium chloride and the solvent was evaporated under reduced pressure at 50°. The residues were dissolved in 2-ml volumes of chloroform.

*Testing the toxicity of extracts to A. salina*

Into separate test-tubes, 100 $\mu$l of concentrated chloroform extracts from mycelium and from the medium and from the original filtrate of the medium were pipetted, allowed to evaporate in a thermostat at 60° and, when cooled, 25 $\mu$l of dimethyl sulphoxide (DMSO) were added to each, thus dissolving the residues. Into the control test-tubes, only 25 $\mu$l of DMSO were added. Into each test-tube, 1 ml of larval suspension was added and then the method of testing the standards of mycotoxins was followed. From the results obtained, the percentage mortality[2] was calculated for the samples investigated.

*Chromatography of extracts and bioautographic detection*

On Silufol sheets, already developed, 10–100 $\mu$l (depending on the percentage mortality) of chosen extracts from mycelium or medium filtrate were applied The sheets with samples were developed in the system[3] in which the separation of the sample of extract was the best. From Silufol sheets, vertical strips with samples 1.5 cm wide were cut out along the whole chromatogram. These strips were cut into segments of 1 cm regardless of the position of the spots and the adsorbent was removed from them into separate test-tubes. For each strip, a control was also prepared: the control contained adsorbent from the square above the front of the chromatogram.

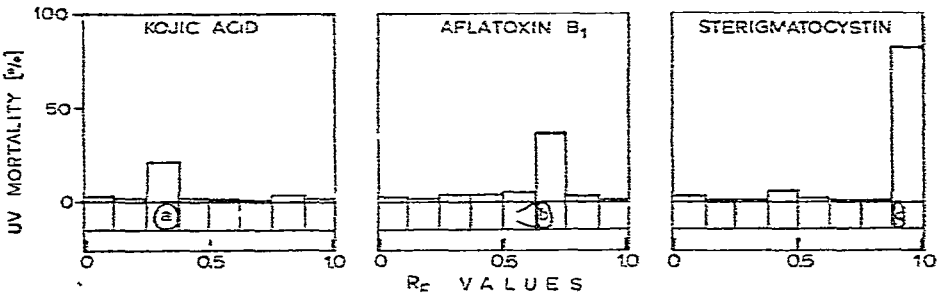


Fig. 1. Bioautographic detection of standard mycotoxins on Silufol sheets in system D. a, Dark yellow spot; b, blue-fluorescing spot; c, red-fluorescing spot.

Into each test-tube with adsorbent, 1 ml of larval suspension was added and then the method for bioautography of the standards of mycotoxins was followed.

*Isolation of unknown active substances from the extract*

For the isolation of an active substance, the extract of filtrate of culture No. S-656 (Aspergillus sp.) was chosen. On the starting point of a plate with an unbound layer of silica gel L, 100–160 $\mu$m (Lachema, Brno, Czechoslovakia), 250 $\mu$l of concentrated chloroform extract obtained from the medium after cultivation of culture S-656 were applied; the plate was developed in system D three times. Fluorescent spots were detected under UV light and the adsorbent from these areas was collected by means of vacuum, eluted with methanol and evaporated to dryness. The residues were dissolved in methanol and a portion of them was tested for toxicity to *A. salina* larvae. The other portion of the residues of individual spots was applied on a Silufol sheet and developed in system D in order to determine the homogeneity of eluates from individual spots. The whole process of the isolation of active substances was repeated on an unbound layer of silica gel L with a larger volume of extract (1.7 ml).

## RESULTS

*Bioautographic detection of the standards of mycotoxins*

Average percentage mortalities for strips from Silufol sheets a, b, c, d, e and f for all squares without mycotoxins ranged from 0 to 9.0%. The average mortality

TABLE I

TOXICITY OF EXTRACTS FROM SELECTED FUNGI TO LARVAE OF *Artemia salina*

| *Culture* | *Taxonomic classification* | *Mortality of larvae (%)* | | |
|---|---|---|---|---|
| | | *Extract of mycelium* | *Extract of medium filtrate* | *Medium filtrate* |
| S-549 | Trichothecium sp. | 100.0 | 100.0 | 63.8 |
| S-556 | Penicillium sp. | 100.0 | 95.1 | 13.5 |
| S-558 | Aspergillus sp. | 79.2 | 77.0 | 58.6 |
| S-562 | *Penicillium camemberti* | 17.5 | 50.0 | 70.5 |
| S-564 | *Penicillium roqueforti* | 27.0 | 74.0 | 2.6 |
| S-569 | *Aspergillus repens* | 41.0 | 65.0 | |

for a square with sterigmatocystin ($R_F = 0.91$ in system D) was 81%, that for a square with aflatoxin ($R_F = 0.65$ in system D) 36% and that for a square with kojic acid ($R_F = 0.33$ in system D) 21% (Fig. 1).

*Testing the extracts of fungi*

The results of testing some extracts from mycelia and medium filtrates after cultivation of fungi, investigated bioautographically, are given in Table I.

*Bioautographic detection of metabolites in the extracts from fungi*

According to the percentage mortality in crude extracts of fungi, chosen ex-

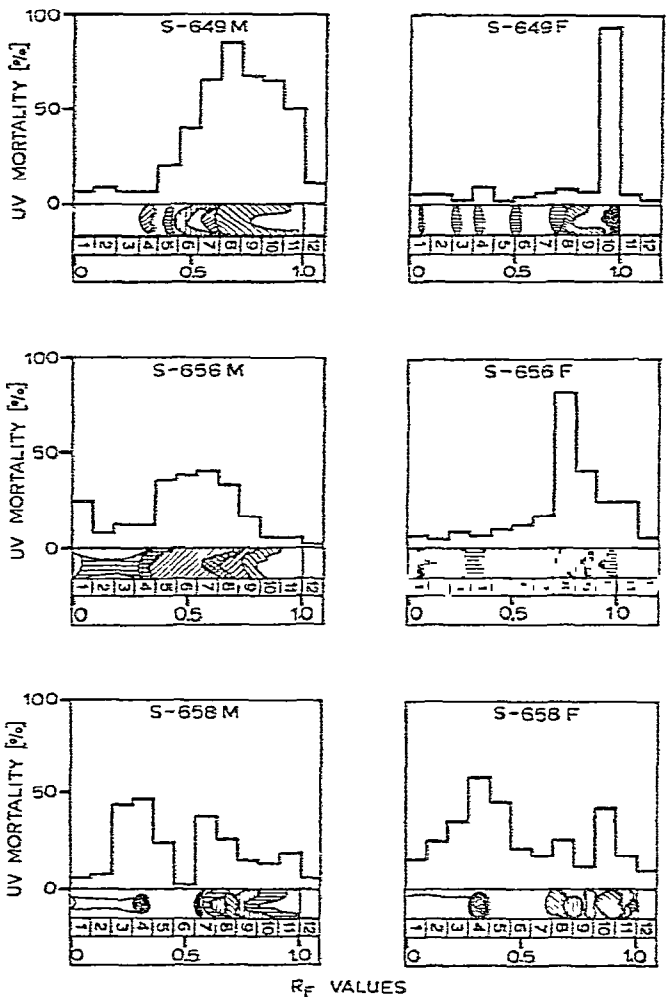


Fig. 2. Evaluation of bioautographic detection in cultures S-649, S-656 and S-658. All chromatograms were developed in system D. Applications on chromatograms: S-649, extract of mycelium 10 $\mu$l and extract of medium filtrate 20 $\mu$l; S-656, extract of mycelium 60 $\mu$l and extract of medium filtrate 60 $\mu$l: S-658, extract of mycelium 20 $\mu$l and extract of medium filtrate 25 $\mu$l. M = extract from mycelium; F = extract from medium filtrate.

tracts were examined by chromatography on Silufol sheets and detected bioautographically. The chromatographic results and the results of bioautographic detection are shown in Fig. 2. On the basis of a chromatographic comparison of the extracts of filtrate and mycelium (in system D) with the standards of aflatoxin $B_1$ ($R_F = 0.67$) and kojic acid ($R_F = 0.33$), it is considered that with culture No. S-658 kojic acid and aflatoxins are produced.

*Isolation of active substances from a chromatogram*

Residues of substances isolated from individual spots on the chromatogram on an unbound layer of silica gel were tested with *A. salina* and the percentage mortality showed that the active substance on the chromatogram was in the region of blue-green and dark green fluorescence (see Fig. 2). Adsorbents from these regions were combined, eluted with methanol and evaporated to dryness. The residues were dissolved in order to determine the toxic effect on *A. salina*. The remaining solution of active substances in methanol was applied on a Silufol sheet along the whole sheet (20 cm) and developed in system D. There were five different fluorescent strips which appeared under UV light. These were removed from the sheet, eluted and evaporated to dryness. From individual spots, the activity of the eluate towards *A. salina* was tested. The active ones were as follows: a pink-fluorescing substance (51% mortality), a green-fluorescing substance (59% mortality) and, closely above it, a yellow substance (85% mortality). These substances were analyzed by the chromatographic systematic analysis[3]. From the results, chromatographic spectra for all three substances were constructed (Fig. 3); these spectra were compared with those of 37 mycotoxins[3] and it was found that the chromatographic spectra of our three substances, produced by culture No. S-656, were different. We are still working on the identification of the unknown metabolites.

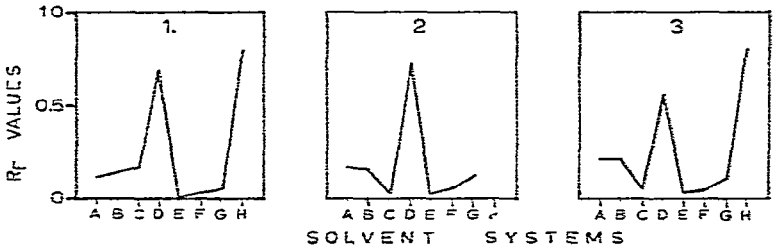


Fig. 3. Chromatographic spectra of isolated substances active towards *A. salina* from culture S-656. 1, Green-fluorescing substance; 2, yellow substance on the chromatogram; 3, pink-fluorescing substance.

## DISCUSSION

Bioautographic detection is used mostly in the study of antimicrobial substances. The simple cultivation of microorganisms provides possibilities for determining the positions of antimicrobial substances on chromatograms. When studying mycotoxins by employing higher animals (mouse, rat, chicken, etc.), a similar detection would not be possible.

In our method of bioautographic detection, the sensitivity of *A. salina* larvae were tested on standard samples of mycotoxins[2]. The advantage of using this model organism for the bioautographic detection is the simplicity of cultivation of the larvae.

For testing the method for the detection of standard mycotoxins, one neutral (D) and one acidic (B) system were used for the development of chromatograms on Silufol sheets. The average percentage natural mortality of larvae in the detection of adsorbent from control squares from Silufol sheets is lower with the neutral system (0–6.7%) than with the acidic system B (0–9.0%).

When employing the acidic system (B), the pH of the medium is not substantially influenced after pouring the adsorbent from the squares cut from chromatograms with larvae cultivation medium.

The possibility of detecting mycotoxins by inserting the whole square from a Silufol sheet into a test-tube (or by cutting it into smaller squares) with larvae suspension was examined. This process did not prove to be suitable as the sharp edges of the aluminium foil damaged mechanically the larvae which had already stopped moving at zero time of testing.

Two types of cultures for testing the extracts for the content of mycotoxins were chosen: (1) fungi of random contaminated foodstuffs and raw materials for foods were isolated; (2) cultures used in the production of some foodstuffs were tested. Wei *et al.*[6] isolated a mycotoxin PR-toxin from the culture *Penicillium roqueforti*. Their culture was isolated, however, from contaminated fodder but, as shown by our results on the toxicity towards *A. salina* of extracts from *P. roqueforti* (S-664) used in the production of cheese, it is necessary to investigate the potential toxigenicity of fungi used in the foodstuffs industry, as the presence of mycotoxins in products can also be caused by used toxicogenic fungi.

The above method for the bioautographic detection of mycotoxins on thin layers by using *Artemia salina* larvae could be applied in screening mycotoxins in contaminated foodstuffs, raw materials for food and fodders owing to its simplicity and reproducibility.

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